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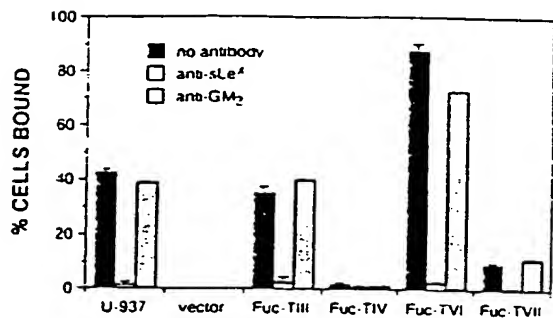


Fig. 6. Adhesion of stably transfected Namalwa KJM-1 cells to soluble E-selectin-coated plates. Namalwa KJM-1 cells were stably transfected with pAMo-FT3, pAMo-FT4, pAMo-FT6, or pAMo-FT7, which directs expression of Fuc-TIII, Fuc-TIV, Fuc-TVI, or Fuc-TVII, respectively, or with the empty vector pAMo. These transfected cells and U-937 cells (for a positive control) were subjected to adhesion assays as described under "Experimental Procedures." Prior to adhesion assays, those cells were incubated with anti-sLe^x mAb KM93 or anti-GM₂ mAb KM696 (as an isotype-matched control) or with no antibody for 30 min at 4 °C. Error bars represent 1 S.D.

sequence alignment and chromosomal localization. Comparison of the amino acid sequences of five α 1.3-Fuc-Ts revealed that the Fuc-TVII sequence had 42–43% and 47% identity with those of the former and latter classes of α 1.3-Fuc-Ts, respectively. PCR analysis using DNAs from hamster-human somatic cell hybrids has shown that the Fuc-TVII gene is located on human chromosome 9. These results indicate that Fuc-TVII is divergent from either class of α 1.3-Fuc-Ts. In addition, these results also deny the identity of Fuc-TVII to either of the sLe^x-synthesizing α 1.3-Fuc-T activities detected in some of the human-mouse leukemic cell hybrids (WGLI-16, -18, and -20) (50) because all of the hybrids lack human chromosome 9.

It is of considerable importance to assign Fuc-TVII to the different α 1.3-Fuc-T activities described to date. Fuc-TVII was shown to fucosylate only a type II acceptor with terminal α 2.3-linked sialic acid among different types of oligosaccharides. This specificity of Fuc-TVII contrasts with those of the other cloned α 1.3-Fuc-Ts. Each of the Fuc-T activities characterized biochemically to date may derive from multiple distinct molecules. Furthermore, the presence of yet unknown Fuc-Ts cannot be excluded. Therefore, Fuc-TVII can be readily assigned to neither of those. Our PCR analyses revealed that both Fuc-TIV and Fuc-TVII were significantly expressed in PMN, monocytes, and their cell lines that have been demonstrated to contain α 1.3-Fuc-T activities for both sialylated and nonsialylated type II acceptors (50–53). Assuming that these activities would arise from a composite of Fuc-TIV and Fuc-TVII, Fuc-TVII may be identical to Fuc-T activity detected in these cells.

As demonstrated here, the competitive PCR method offers a sensitive quantitation of specific transcripts across a wide range of amounts. Assuming that variations in conversion of mRNA into cDNA would be very small among different mRNA species, normalization with the level of a housekeeping gene such as β -actin should allow precise comparisons of transcription levels. When using reverse-transcribed cDNAs, either different transcriptional initiations or alternative splicing events may obstruct the precise quantitation. With respect to the α 1.3-Fuc-T genes tested, such initiations and events should be precluded because no intron has been identified in the regions surrounded by the respective sets of PCR primers (20, 22–25). In some cases, contamination of chromosomal DNA may cause troubles against quantitation of target cDNAs. Taking into consideration that all of the RNA samples analyzed in this study were prepared by the same procedure, and judging from the

results in Table III, the levels of target genes resulting from contaminated chromosomal DNA should be below 0.01% relative to the level of β -actin mRNA.

As mentioned above, a number of conflicting results have been reported on the participation of Fuc-TIV in the synthesis of the sLe^x determinants (21–23, 49, 50). As shown here, the expression of Fuc-TIV in Namalwa KJM-1 cells increased the level of antigens recognized by anti-sLe^x mAb CSLEX-1, albeit not by the other two anti-sLe^x mAbs. Furthermore, the cells transfected with the Fuc-TIV gene exhibited virtually no binding to soluble E-selectin in spite of its efficient expression. A possible explanation for these discrepant results is that the CSLEX-1-reactive antigen whose level was increased by Fuc-TIV expression may not be the sLe^x determinant. Another explanation may be a difference in the Fuc-TIV mRNA level as discussed previously (22, 23) and is apparently consistent with a slight increase in the sLe^x level by the enforced expression of Fuc-TIV in Namalwa KJM-1 cells. We have discussed previously that an α 2.3-sialyltransferase ST-4 directs *de novo* synthesis of the sLe^x determinant *in vivo*. Our further analysis of the *in vitro* enzymatic properties of ST-4 has revealed that ST-4 sialylates *in vitro* the nonreducing termini of Le^x-related oligosaccharides such as LNFP-III, although much less efficiently than nonfucosylated type II acceptors, to yield the sLe^x determinants.³ With this observation in consideration, the Fuc-TIV-mediated sLe^x formation may result from subterminal fucosylation of type II chains by Fuc-TIV followed by their terminal sialylation with ST-4. Therefore, to resolve these conflicts reported, it will be required to analyze the expression levels of other glycosyltransferases including ST-4 in the cell lines of concern.

Alternatively, differences in the amounts of precursors of the selectin carbohydrate ligands or specific scaffold proteins (or glycolipids) carrying those ligands should be taken into consideration. Expression of either Fuc-TVI or Fuc-TVII remarkably increased the levels of the sLe^x determinants. However, substantial distinctions in the E-selectin binding and *M. amurensis* lectin I binding capacities of Fuc-TVI and Fuc-TVII have been observed after their transfection into Namalwa KJM-1 cells, suggesting the existence of significant differences between these Fuc-Ts in the *in vivo* acceptor specificities for oligosaccharides and/or in the expression levels of scaffolds presenting the selectin ligands. In this context, it will be of special interest that a ligand for P-selectin has been demonstrated to be a specific glycoprotein carrying a small fraction of the total sLe^x sequences on neutrophil membranes (54) and has been cloned very recently (55). In addition, a ligand on myeloid cells which binds with high affinity to E-selectin has been reported to be a 150-kDa glycoprotein (56). Taking these observations into account, the difference in the E-selectin binding capacity of the Fuc-TVI- and Fuc-TVII-transfected cells observed here may derive from that in the levels of such scaffold proteins and/or specific sLe^x structures.

In conclusion, this study has provided a number of informative results on the α 1.3-Fuc-Ts participating in the biosynthesis of the sLe^x determinants. The *in vivo* ability of Fuc-TVII to synthesize the sLe^x epitopes that bind to E-selectin, together with the restricted expression of Fuc-TVII in leukocytes, suggest that among five cloned α 1.3-Fuc-Ts, Fuc-TVII is the most promising candidate involved in the biosynthesis of the selectin ligands; further studies using gene disruption and/or antisense technology will be necessary to obtain more definitive evidence for the identification of Fuc-T(s) participating in that biosynthesis.

³ K. Sasaki, Y. Amino, and T. Nishi, unpublished observations.

⁴ K. Sasaki, K. Kawashima, and T. Nishi, unpublished results.

TABLE II
Acceptor substrate specificity of Fuc-TVII

Acceptor	Activity	
	pmol/min/mg protein	pmol/min/mg protein
NeuAc α 2-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc	α 2,3-sialyl LNT ¹	3.2
Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc	(LNT)	0*
NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc	α 2,3-sialyl LNT ¹	0
Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc	(LNT)	0
Fuc α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc	(LNFP-I)	0
NeuAc α 2-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc	α 2,3-sialyl LNFP-V ¹	0
3		
Fuc α 1		
Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc	(LNFP-V)	0
3		
Fuc α 1		

* A value of 0 indicates less than 0.1 pmol/ml of medium/h.

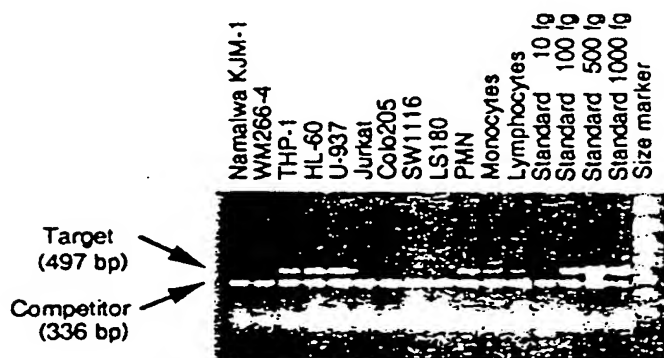


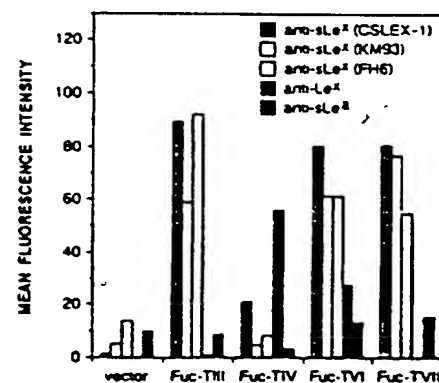
FIG. 4. Competitive PCR analysis of Fuc-TVII transcripts in various cells. Single-stranded cDNAs reverse-transcribed from total RNA of a variety of cells labeled on the top or standard Fuc-TVII cDNAs (10, 100, 500, and 1,000 fg) were mixed with 100 fg of the competitor cDNA (truncated Fuc-TVII cDNA) and subjected to 28 cycles of PCR as described under "Experimental Procedures." The amplified products were separated by electrophoresis in a 1.8% agarose gel and visualized by staining with ethidium bromide. Size markers, from top: 4.3, 1.3, 1.1, 0.68, 0.39, 0.25, and 0.12 kb.

TABLE III
Quantitation of α 1,3-Fuc-T transcripts in a variety of cells by competitive PCR analyses

Expression levels of five α 1,3-Fuc-Ts in a variety of the cells listed were examined as described under "Experimental Procedures." The levels in PMN, monocytes, and lymphocytes are expressed as the averages of data derived from three independent RNA preparations from two healthy adult donors.

Cell	Expression levels of α 1,3-Fuc-Ts relative to the level of β -actin				
	Fuc-TIII	Fuc-TIV	Fuc-TV	Fuc-TVI	Fuc-TVII
HL-60	<0.01	1.3	<0.01	0.02	0.32
U-937	<0.01	0.48	<0.01	<0.01	0.59
THP-1	<0.01	0.23	<0.01	<0.01	0.20
Jurkat	<0.01	<0.01	0.01	0.01	<0.01
Namalwa KJM-1	<0.01	<0.01	<0.01	0.03	<0.01
PMN	<0.01	0.04	<0.01	<0.01	0.77
Monocytes	<0.01	0.16	<0.01	<0.01	0.06
Lymphocytes	<0.01	0.12	<0.01	<0.01	0.08
WM266-4	<0.01	0.04	<0.01	<0.01	<0.01
Colo205	2.0	0.20	0.01	1.1	<0.01
SW1116	1.7	0.56	<0.01	0.04	<0.01
LS180	2.4	0.31	<0.01	0.75	0.03

encoding other mammalian glycosyltransferases. In a previous study (18), we have described the expression cloning of an α 2,3-sialyltransferase ST-4 that directs *de novo* expression of the sLe^x determinant, using lectin resistance selection. In our

FIG. 5. Flow cytometry histograms of Namalwa KJM-1 cells stably transfected with α 1,3-Fuc-T expression plasmids. Namalwa KJM-1 cells were stably transfected with plasmid pAmo-FT3, pAmo-FT4, pAmo-FT6, or pAmo-FT7, which directs expression of Fuc-TIII, Fuc-TIV, Fuc-TVI, or Fuc-TVII, respectively, or with the empty vector pAmo. These cells were stained with the mAbs listed in the inset and subjected to flow cytometry analysis as described under "Experimental Procedures." The data presented here are the mean fluorescence intensities of the entire population of these transfected cells. The mean fluorescence intensities were obtained by subtracting those of responding cells stained with normal BALB/c mouse serum.

initial efforts to clone novel Fuc-T(s) involved in the biosynthesis of the sLe^x determinants, we applied the cloning approach with *M. amurensis* lectin I resistance, which selected a Fuc-TV clone. Our analysis with FACS showed that the great majority of Namalwa KJM-1 cells lost the *M. amurensis* lectin I binding capacity by Fuc-TVI expression, whereas that capacity was unchanged by Fuc-TVII expression.² This explains the inability to enrich Namalwa KJM-1 cells expressing Fuc-TVII with *M. amurensis* lectin I resistance and suggests the presence of a difference(s) in the *in vivo* acceptor specificity between Fuc-TVII and Fuc-TVI. Although the binding specificity of *M. amurensis* lectin I has not been reported, *M. amurensis* lectin I is likely specific to galactosyl residues, especially the Gal β 1-3GlcNAc structure.³ This specificity of *M. amurensis* lectin I, however, does not simply account for the selection of a Fuc-TV clone. Further analyses are currently under way to determine the reason for the acquisition of the *M. amurensis* lectin resistance phenotype.

As pointed out by Weston *et al.* (25), four cloned α 1,3-Fuc-Ts can be divided into two classes: chromosome 19-localized α 1,3 fucosyltransferases (Fuc-TIII, Fuc-TV, and Fuc-TVI) and chromosome 11-localized Fuc-TIV, based on the results of their se-

² K. Sasaki and T. Nishi, unpublished observations.³ Information from Vector Research Laboratories.

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Fig. 2. Nucleotide and deduced amino acid sequences of cDNA insert in pAmo-FT7. The putative transmembrane domain is boxed. Two potential N-glycosylation sites are indicated by closed circles. A possible polyadenylation signal is underlined.

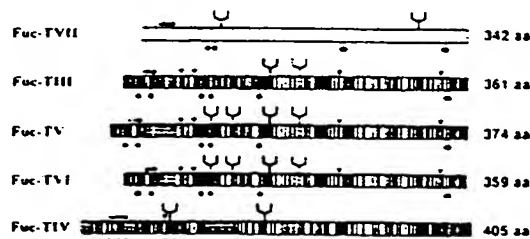


Fig. 3. Schematic comparisons of the primary structures of the five cloned human $\alpha 1,3$ -Fuc-Ts. Amino acid sequence identities were determined by comparison of the Fuc-TVII sequence with the other sequences. The residues differing from the Fuc-TVII template and the inserted residues relative to that template are indicated by solid and dashed vertical bars, respectively. Closed triangle denote the lack of the corresponding residues relative to the Fuc-TVII sequence. Putative transmembrane domains, potential N-glycosylation sites, and cysteine residues are indicated by shaded thick bars, stick figures, and bars with a closed circle, respectively. The sizes of the predicted polypeptides are shown approximately to scale.

been published about the capability of Fuc-TIV to synthesize the sLe^x determinants in leukocyte-derived hosts, it will be of importance to analyze such capability in Namalwa KJM-1 cells. As controls, we attempted to perform such analyses on Fuc-TIII and Fuc-TVI expressed abundantly in the colon adenocarcinoma cell lines. In addition, it will be of considerable interest to confirm that Fuc-TVII exhibits the restricted acceptor specificity *in vivo*. To address these issues, Namalwa KJM-1 cells transfected with pAmo-FT3, pAmo-FT4, pAmo-FT6, pAmo-FT7, or their parental vector pAmo were examined for changes in surface expression of the sLe^x, Le^x, sLe^a, and Le^a antigens by flow cytometric analyses using their respective mAbs. None of these plasmids changed the level of Le^a antigen by their transfection. As shown in Fig. 5, Fuc-TVII expression increased all of the levels of sLe^x epitopes recognized by anti-sLe^x mAbs CSLEX-1, KM93, and FH6, whereas neither the Le^x nor the sLe^a level was changed significantly by Fuc-TVII expression, reflecting the *in vitro* acceptor specificity of Fuc-TVII. Fuc-TIV expression elevated the level of the sLe^x epitope recognized by only CSLEX-1 among three anti-sLe^x mAbs tested (Fig. 5), consistent with the result of Fuc-TIV expression in

CHO cells reported by Meier *et al.* [49]. Changes in the levels of these epitopes by Fuc-TIII expression were quite similar to those caused by Fuc-TVII expression (Fig. 5). It should be of note that Fuc-TIII expression was incapable of increasing the level of either Le^x, Le^a, or sLe^a antigens in Namalwa KJM-1 cells in contrast to their increased appearance in Fuc-TIII-transfected CHO and COS cells [20, 22, 24, 25]. As presented in Fig. 5, Fuc-TVI expression increased the levels of sLe^x and Le^x epitopes but not the sLe^a level.

Namalwa KJM-1 Cells Expressing Fuc-TVII Bind to E-Selectin—To determine whether Fuc-TVII and/or Fuc-TIV mediates the formation of the sLe^x determinant functioning in E-selectin binding, Namalwa KJM-1 cells transfected with pAmo-FT7 or pAmo-FT4 were tested for their ability to bind to soluble E-selectin immobilized on 96-well microtiter plates. As controls, the cells transfected with pAmo, pAmo-FT3, or pAmo-FT6 were also examined. U-937 cells adhered in a dose-dependent manner to soluble E-selectin until a plateau was reached at ~0.2 μ g/well (data not shown). The results of adhesion assays using a fixed amount (0.2 μ g/well) of soluble E-selectin are shown in Fig. 6. Fuc-TVII transfectants were found to bind to soluble E-selectin, although the binding capacity of the Fuc-TVII transfectants was lower than those of the Fuc-TIII and Fuc-TVI transfectants and U-937 cells. By contrast, the Fuc-TIV transfectants exhibited virtually no binding to soluble E-selectin (Fig. 6). Their adhesion, except for that of Fuc-TIV transfectants, was inhibited completely by anti-sLe^x mAb KM93 but not with a control mAb KM696 (Fig. 6). These results, together with those of the expression patterns of five $\alpha 1,3$ -Fuc-Ts, suggest that Fuc-TVII may be involved in the biosynthesis of the selectin ligands in leukocytes.

DISCUSSION

In this study, we have enriched stable transfectants highly expressing the sLe^x antigens with FACS to identify a novel $\alpha 1,3$ -Fuc-T (Fuc-TVII) that may participate in the biosynthesis of the selectin carbohydrate ligands. This is the first demonstration of expression cloning of glycosyltransferase cDNA with FACS. Furthermore, as described under "Experimental Procedures," this cloning approach allowed us to isolate a Fuc-TIII clone and will be therefore useful for the isolation of cDNAs

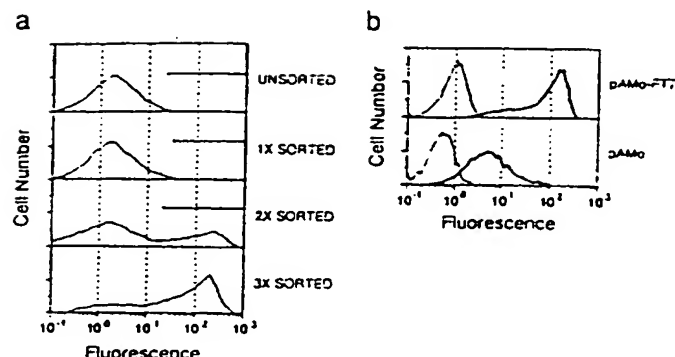


Fig. 1. Expression cloning of Fuc-TVII by FACS. Panel a, Namalwa KJM-1 cells stably transfected with the THP-1 cDNA library were stained with the anti-sLe^x mAb KM93 and were subjected to three rounds of sorting with FACS. The cells with fluorescence intensities indicated by the bars were collected and subjected to the subsequent sorting. Panel b, flow cytometric analysis of the cells transfected with pAMo-FT7 (upper panel) or the empty vector pAMo (lower panel) after staining with mAb KM93 (bold lines) or normal BALB/c mouse serum (thin lines).

leukocytes. In parallel with this approach, we sought to amplify such Fuc-T sequences by the PCR cloning approach using degenerated primers based on two consensus sequences (YKFY-LAFEN and FIHVDDF) conserved among Fuc-TIII, Fuc-TIV, and Fuc-TV (20, 22, 24) but failed to identify such novel sequences. Assuming that such novel Fuc-Ts would increase the level of sLe^x antigens in hosts of leukocyte origin, we therefore chose a cloning strategy to enrich the clones stained brightly with anti-sLe^x mAbs by use of a fluorescence-activated cell sorter (FACS). Namalwa KJM-1 cells were transfected stably with the THP-1 cDNA library and subjected to sorting on a FACS with anti-sLe^x mAb KM93. Two rounds of sorting split the cell population into two types of cells which expressed high and low levels of the sLe^x antigens (Fig. 1a). After the third sorting, the great majority of the cell population was shown to be enriched for high sLe^x expression (Fig. 1a, the bottom panel). Individual plasmids were rescued from the clones sorted thirdly and then examined for their ability to increase the level of the sLe^x antigens on Namalwa KJM-1 cells, which identified a plasmid pAMo-FT7 directing *de novo* expression of the sLe^x antigens (Fig. 1b).

cDNA Sequence Predicts a Protein with Homology to α 1,3-Fuc-Ts.—DNA sequencing analysis revealed that the cDNA insert in pAMo-FT7 (~1.7 kb long) contained a single long open reading frame encoding a protein of 342 amino acids with an *M_r* of 39,244 (Fig. 2). The initiation codon was determined based on the rules of Kozak for mammalian translation initiation (48). The deduced primary sequence predicts that this protein has a type II transmembrane topology, as has been found for all other glycosyltransferases cloned to date. Comparison of this primary sequence with other amino acid sequences in DNA and protein data banks revealed strong similarities to the sequences of four α 1,3-Fuc-Ts cloned to date (Fig. 3). This sequence has 42–43% sequence identity with either the Fuc-TIII, Fuc-TV, or Fuc-TVII sequence and 47% identity with the Fuc-TIV sequence (Fig. 3), suggesting that this protein belongs to the α 1,3-Fuc-T family. Furthermore, to determine the chromosomal location of this gene, PCR analysis was performed using a series of DNAs from hamster-human somatic cell hybrids. The predicted fragment of 218 bp was amplified only when genomic DNAs from the hybrids containing human chromosome 9 were used, indicating that this gene is located on chromosome 9.

Cloned cDNA Encodes an α 1,3-Fuc-T.—To prove that this

protein (Fuc-TVII) contains a Fuc-T activity, we expressed and purified that putative catalytic domain (amino acids 39–342) as a protein A fusion protein, but we failed to detect any significant Fuc-T activity with this fusion. By contrast, the protein A-Fuc-TVII fusion was found to exhibit considerable activity (data not shown), although the reason for this discrepancy remained unsolved. Alignment of the sequence of Fuc-TVII with that of Fuc-TVII revealed that Fuc-TVII had an insertion of 15 amino acids downstream of the transmembrane region relative to Fuc-TVII. Based on the result, plasmid pAMoA-FT7 encoding another protein A-Fuc-TVII fusion (designated A6FT7) was constructed by inserting this peptide of Fuc-TVII between the protein A portion and the COOH-terminal portion (amino acids 39–342) of Fuc-TVII and was expressed in Namalwa KJM-1 cells. When α 2,3-sialyl LNT was used as an acceptor, a significant amount of α 1,3-Fuc-T activity bound to IgG-Sepharose was detected in the medium of Namalwa KJM-1 cells transfected with pAMoA-FT7, whereas no activity was detected for either LNT, LNT, LNFP-I, LNFP-V, α 2,3-sialyl LNT, or α 2-sialyl LNFP-V (Table II). Moreover, in the medium of the cells transfected with the protein A fusion vector pAMoA (18) or plasmid pAMo-FT7, no activity bound to IgG-Sepharose was detected for either of these acceptors. These results indicate that the protein A6FT7 fucosylates specifically a type II oligosaccharide with terminal α 2,3-linked sialic acid. In addition, the analyses for identification and confirmation of the reaction products revealed that the protein A6FT7 attached fucose in α 1,3-linkage to the *N*-acetylglucosamine residue of sialyl LNT to form the sLe^x determinant. Taken together, the cloned cDNA was demonstrated to encode an α 1,3-Fuc-T that had more restricted acceptor requirements than the other four α 1,3-Fuc-Ts (20–26).

Determination of Expression Levels of Five Cloned α 1,3-Fucosyltransferases in a Variety of Cells.—The sLe^x determinants on PMN and monocytes were demonstrated to serve as ligands for selectins on vascular endothelial cells and platelets. In addition, those determinants were suggested to contribute to the metastatic behavior of carcinoma cells. However, it is unclear which α 1,3-Fuc-Ts are involved in each biological process. To address this question, we sought to examine their expression levels in a variety of cells by quantitative PCR analyses using the respective sets of primers listed in Table I. A typical result of such analysis for Fuc-TVII mRNA is shown in Fig. 4. Data of the expression levels of five α 1,3-Fuc-Ts were compiled and are summarized in Table III. Both Fuc-TIV and Fuc-TVII genes were significantly expressed in PMN, monocytes, and their immortalized cell lines (HL-60, U-937, and THP-1), which were shown to present the selectin ligands constitutively. In addition, the Fuc-TIV gene was expressed considerably in a variety of cells, whereas the expression of Fuc-TVII was restricted in leukocytes, especially in PMN (Table III). Such further analyses were performed with human umbilical vein endothelial cells and other human cell lines derived from a variety of tissues such as KATO III (gastric carcinoma), Capan-1 (pancreas carcinoma), PC-3 (prostate carcinoma), SK-N-MC (neuroblastoma), PC-9 (lung carcinoma), and HeLa (cervix carcinoma). As a result, the expression level of Fuc-TVII was shown to be below 0.01% relative to the β -actin level in all of these cells and cell lines. In light of the previous observations on cancer metastasis (1, 14–17), it should be noteworthy that both Fuc-TIII and Fuc-TVII genes were highly expressed in colon adenocarcinoma cell lines Colo205, SW1116, and LS180, except for the poor expression of Fuc-TVII in SW1116. Taken collectively, both Fuc-TIV and Fuc-TVII were shown to be candidates participating in the biosynthesis of the sLe^x determinants in leukocytes.

Either Fuc-TIV or Fuc-TVII Directs Biosynthesis of the sLe^x Determinants in Namalwa KJM-1 Cells.—Since no report has

TABLE 1
Oligonucleotide primers used for competitive PCR analyses

RNA	Forward primer	Reverse primer	Size of amplified DNA	
			Target	Competitor
Fuc-TIII	5'-CCTCCCGACAGGACACCCTCC-3'	5'-GCGTCCGTACACGTCACCTTG-3'	521	349
Fuc-TIV	5'-GAGAGGCTCAGGCCGTGCTTT-3'	5'-GCAGGAGCCCAATTTCGGGCAC-3'	516	345
Fuc-TV	5'-GAACCTGTACACGGGGCTCCC-3'	5'-GCGTCCGTACACGTCACCTTG-3'	555	405
Fuc-TVI	5'-AATGGGTCCCGCTTCCCAGACAG-3'	5'-GCGTCCGTACACGTCACCTTG-3'	534	355
Fuc-TVII	5'-CACCTCCGAGGCATCTTCAACTG-3'	5'-CGTTGGTATCGGCTCTCATTTCATG-3'	497	336
β -Actin	5'-GATATCGCCGCGCTCGTCGTCGAC-3'	5'-CAGGAAGGAAGGCTGGAAGAGTGC-3'	789	639

total volume of 30 μ l. Acceptors lacto-N-neotetraose (LNnT), lacto-N-tetraose (LNT), lacto-N-fucopentaose I (LNFP-I), lacto-N-fucopentaose V (LNFP-V), and α 2,3-sialyl LNT were purchased from Oxford Glyco-systems. Acceptors α 2,3-sialyl LNnT and α 2,3-sialyl LNFP-V were prepared by sialylation of LNnT and LNFP-V with protein A-fused ST-4 (18) and protein A-fused human CMP-N-acetylneuraminase-V:acetylactosaminide α 2,3-sialyltransferase (18, 42), respectively. These acceptors were pyridylaminated according to the method of Kondo *et al.* (43). After incubation at 37 °C for 2 h, the reactions were terminated by boiling for 5 min. After centrifugation of the reaction mixtures at 12,000 \times g for 10 min, an aliquot of the supernatant was subjected to HPLC analysis using a TSK-gel ODS-80T_M column (4.6 \times 300 mm; Tosoh). The reaction products were eluted with a 20 mM ammonium acetate buffer (pH 4.0) at a flow rate of 1.0 ml/min at 30 °C and were monitored with a fluorescence spectrophotometer (Hitachi model F-1100). Parallel reactions were done in the absence of GDP-fucose to identify products and to check substrate and product hydrolysis.

Enzyme activity is defined as pmol of acceptor substrate fucosylated/ml of culture medium/h. The amounts of products were determined from their fluorescence intensities using pyridylaminated lactose as a standard. The reaction products were identified by comparison of their retention times on HPLC with those of the following pyridylaminated standard oligosaccharides: LNFP-II, LNFP-III, LNFP-V, α 2,3-sialyl LNFP-II (with the sLe^x structure), α 2,3-sialyl LNFP-III (with the sLe^x structure), and α 2,3-sialyl LNFP-V. Pyridylaminated α 2,3-sialyl LNFP-II and pyridylaminated α 2,3-sialyl LNFP-III were prepared by fucosylation of pyridylaminated α 2,3-sialyl LNT and pyridylaminated α 2,3-sialyl LNnT with the purified Fuc-TIII and Fuc-TVI enzymes fused with protein A, respectively.

Quantitation of Fuc-T Transcripts Using Competitive PCR—The levels of Fuc-TIII, Fuc-TIV, Fuc-TV, Fuc-TVI, and Fuc-TVII transcripts were measured by competitive PCR (44) using their respective cDNAs, which were reverse-transcribed from RNA samples. For distinction of a target cDNA from its competitor DNA, plasmids pUC119-FT3d, pUC119-FT4d, pUC119-FT5d, pUC119-FT6d, and pUC119-FT7d, which carried a small deletion within the respective cDNAs, were prepared by deleting the 172-bp *Tth*1111-*Mbo*II fragment of Fuc-TIII cDNA from pUC119-FT3R, the 171-bp *Sma*I-*Stu*I fragment of Fuc-TIV cDNA from pUC119-FT4, the 150-bp *Eco*RV-*Mbo*II fragment of Fuc-TV cDNA from pUC119-FT5, the 179-bp *Ban*I-*Pvu*II fragment of Fuc-TVI cDNA from pUC119-FT6R, and the 161-bp *Apa*I-*Bst*XI fragment of Fuc-TVII cDNA from pUC119-FT7, respectively. Plasmids pUC119-FT3R and pUC119-FT6R were constructed by inserting the 1.0-kb *Hind*III (blunt)-*Asp*¹ (blunt) fragment of pAmo-FT3 and the 2.1-kb *Eco*RV-*Sac*I (blunt) fragment of pAmo-FT6, respectively, into the *Hinc*II site of pUC119. To preclude sample-to-sample variations, the level of β -actin mRNA was measured for each cDNA sample by PCR using plasmid pUC119-ACTd as a competitor. Plasmid pUC119-ACTd carrying a truncated human β -actin cDNA was generated by deleting the 150-bp *Eco*O109I-*Bst*EII fragment from a β -actin cDNA that was amplified from the single-stranded cDNAs of U-937 by the PCR using primers 5'-aagtataagcttCATGGATGATGATATCGCCGCGCTCGT-3' and 5'-atttaaggtacGAA-GCATTTCGGGTGGACGATGGAGGGG-3'. All of the plasmid DNAs were subjected to PCR analyses after their linearization with restriction enzyme digestion.

From total RNA (5 μ g for cell lines or 1 μ g for human blood leukocytes) prepared by the guanidine isothiocyanate/CsCl method (45), cDNAs were synthesized using oligo(dT) primers in a total volume of 21 μ l and diluted either 50-fold (for cell lines) or 10-fold (for blood leukocytes) with H₂O. Ten μ l of this dilution was subjected to the two-step quantitative PCR analysis that consisted of the first PCR for rough measurement using 10 fg of linearized competitor DNAs or 10 pg of pUC119-ACTd and the second PCR for accurate quantitation based on the

amount of target cDNAs estimated by the first PCR. After incubation at 97 °C for 5 min, PCR was performed in a final volume of 40 μ l using primers (0.5 μ M each) listed in Table 1 by 15–19 cycles for β -actin, 23–32 cycles for Fuc-Ts except Fuc-TV (of 94 °C for 30 s, 65 °C for 1 min, and 72 °C for 2 min). The PCR for Fuc-TV was done by 27–30 cycles of 94 °C for 30 s and 72 °C for 4 min. The PCR for Fuc-TIV and Fuc-TVI was performed in the presence of 5% dimethyl sulfoxide. After amplification, 15- μ l aliquots were subjected to electrophoresis in 1.8% agarose gels, followed by photographic recording of the gels stained with ethidium bromide. Amplified DNA fragments were quantified by scanning the negative films using a densitometer (Shimadzu model C-9000). The amounts of amplified target cDNAs were calculated from their respective standard curves, converted into the values of mol numbers, and normalized by those for β -actin.

Construction and Purification of Soluble E-Selectin—According to an approach similar to that described by Lobb *et al.* (46), we prepared soluble form of human E-selectin protein lacking the transmembrane and cytoplasmic portions. As an attempt to facilitate its purification, we added an avidin-binding peptide of 8 amino acids (AGHPQGP) (47) to the COOH terminus of truncated E-selectin (amino acids 1–531), but the resulting soluble E-selectin was found not to bind to an avidin affinity column in that purification process. A fragment encoding the truncated E-selectin (amino acids 1–531) was generated from plasmid BBG37 carrying a full-length of E-selectin cDNA by a combination PCR amplification with restriction enzyme digestion and was cloned into a mammalian expression vector pAGE248 (a gift from Dr. T. Mizukami) together with double-stranded oligonucleotides encoding the avidin binding sequence, which formed a plasmid pSELAM248A expressing the modified soluble E-selectin. The vector pAGE248 is a derivative of pSE18d2-4 (30) in which the segments of SV40 early promoter, interferon- β cDNA, and G418 resistance unit were replaced with those of Moloney murine leukemia virus promoter, multicloning site, and hygromycin resistance unit, respectively. After transfection of Namalwa KJM-1 cells with pSELAM248A, a clone E1M1 producing \sim μ g/ml of the soluble E-selectin was obtained using the dihydrofolate reductase gene coamplification method as described by Miyaji *et al.* (30). From the serum-free conditioned medium of clone E1M1, the soluble E-selectin protein was purified by the same procedure as that of Lobb *et al.* (46) except for omission of Mono Q column chromatography. In this purification procedure, we used anti-E-selectin mAb KM994 for immunoaffinity column chromatography.

Cell Adhesion Assays—Assays to determine adhesion of transfected Namalwa KJM-1 cells and U-937 cells to soluble E-selectin-coated 96 well microtiter plates were performed as described by Lobb *et al.* (46). Before adding these cells to the wells, the cells were incubated with 10 μ g/ml KM93 or a control mAb KM696 (for testing the antibody-mediated inhibition of cell binding) or without mAb for 30 min at 4 °C. Cell adhesion was quantified by counting the number of adherent cells after detaching them from the plates with trypsin treatment.

RESULTS

Expression Cloning of cDNA Directing Biosynthesis of the sLe^x Determinants—To search novel Fuc-Ts involved in the biosynthesis of the selectin carbohydrate ligands, we employed the expression cloning approach using lectin resistance selection described previously (18), and achieved the isolation of a Fuc-TVI clone from the SW1116 cDNA library by selection with the cytostatic *M. amurensis* lectin I. As shown below, our analysis revealed that Fuc-TVI was not substantially expressed in leukocytes presenting the selectin ligands, indicating that Fuc-TVI is not involved in the biosynthesis of those ligands in

the participation of yet unknown α 1.3-Fuc-Ts), which may resolve most of the apparent conflicts.

In this study, we report the expression cloning of a novel human α 1.3-Fuc-T, designated Fuc-TVII, which directs the biosynthesis of the sLe^x determinant in the human Burkitt lymphoma cell line Namalwa. Quantitative analyses of the expression levels of five cloned α 1.3-Fuc-Ts in various cells revealed that both Fuc-TIV and Fuc-TVII were significantly expressed in myeloid lineage cells. Either Fuc-T was found to form the sLe^x antigens at the cell surface, when expressed in Namalwa cells. Fuc-TVII expression has conferred considerable E-selectin binding ability on the cells in contrast to almost no binding activity given by Fuc-TIV expression, suggesting that Fuc-TVII may participate in the biosynthesis of the selectin ligands.

EXPERIMENTAL PROCEDURES

Nomenclature of Sialyl- and Fucosyltransferases—To simplify discussion, four members of the cloned human α 1.3-Fuc-Ts will be called Fuc-TIII, Fuc-TVI, Fuc-TV, and Fuc-TV according to the designation of Lowe and co-workers [24, 25]. A novel α 1.3-Fuc-T identified in this study will be referred to as Fuc-TVII. The Gal β 1-3/1-4GlcNAc α 2.3-sialyltransferase described previously [18] will be called ST-4.

Cell Lines—Namalwa KJM-1, a subline of the human Burkitt lymphoma cell line Namalwa, was cultivated in serum-free RPMI 1640 medium as described [27, 28]. Cell lines WM266-4, THP-1, HL-60, U-937, Colo205, and LS180 were obtained from the American Type Culture Collection. SW1116 and T-cell line Jurkat were gifts from Dr. Toshitada Takahashi (Aichi Cancer Center). These cell lines were cultured in RPMI 1640 medium containing 10% fetal calf serum.

Preparation and Fractionation of Blood Leukocytes—Human polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells were separated from the peripheral blood of healthy adult donors using PolymorphprepTM (Nycomed Pharma). The peripheral blood mononuclear cells were adhered to plastic Petri dishes and separated into adherent monocyte-enriched and nonadherent lymphocyte-enriched populations [29].

Construction of cDNA Libraries—Double-stranded cDNAs were synthesized with oligo(dT) primers using poly(A)⁺ RNA prepared from THP-1 or SW1116 cells. The cDNA libraries were constructed by inserting blunt ended cDNA larger than 1.6 kilobases (kb) between two SfiI sites of the expression vector pAmo [18] with the help of adaptors formed by oligonucleotides 5'-CTCTAAAG-3' and 5'-CTTTAGAGCAC-3'.

Monoclonal Antibodies and Flow Cytometric Analysis—Anti-E-selectin mAb KM994 was generated by immunizing BALB/c mice with the crude membrane fractions prepared from CHO cells that express human E-selectin highly. The cells were generated by transfection with pSEdELAM1 that was constructed by inserting an E-selectin cDNA of plasmid BBG57 (British Biotechnology) into a vector pSE1 β d2-4 a gift from Dr. T. Mizukami, Kyowa Hakko Tokyo Research Laboratories, Ref. 30). The mouse anti-sLe^x mAb KM93 (IgM), the mouse anti-sLe^x mAb KM231 (IgG1), the mouse anti-Lewis x (Le^x) (Gal β 1-4Fuc α 1-3)GlcNAc mAb KM380 (IgM), and the mouse anti-ganglioside G_{M2} mAb KM696 (IgM) were prepared as described [31-34]. The mouse anti-sLe^x mAb CSLEX-1 (IgM) [35] was purified from the supernatants of hybridoma HB8580 obtained from the American Type Culture Collection. The mouse anti-Lewis x (Le^x) (Gal β 1-3(Fuc α 1-4)GlcNAc) mAb was obtained from the International Reagent Co. (Kobe). The mouse anti-dimeric sLe^x (NeuAc α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc β 1-3Gal β 1-4Fuc α 1-3)GlcNAc mAb FH6 (IgM) [36, 37] was a gift from Dr. Reiji Kannagi (Aichi Cancer Center). Transfected Namalwa KJM-1 cells were stained with each of these mAbs or with BALB/c mouse normal serum (as a control) and were analyzed on an EPICS Elite flow cytometer (Coulter Electronics Inc.) as described [18].

DNA Sequencing—DNA Sequences were determined by the dideoxynucleotide chain termination method [38] using the Applied Biosystems model 373A DNA Sequencer.

Expression Cloning of cDNA Encoding Fuc-TVII—Namalwa KJM-1 cells were transfected with the THP-1 cDNA library by electroporation as described [39] and grown for 24 h. Stably transfected cells were selected by cultivation for >14 days in the presence of G-418 (0.5 mg/ml). The cells were stained with the mAb KM93 followed by staining with fluorescein isothiocyanate-conjugated goat anti-mouse IgG/IgM. The cells highly expressing the sLe^x antigens were enriched by three rounds

of sorting on the flow cytometer. Plasmid DNAs were recovered from these cells as described [40]. Individual plasmids were examined for their ability to increase the level of sLe^x antigens in Namalwa KJM-1 cells to select a Fuc-TVII-expressing clone named pAmo-FT7.

Expression Cloning of cDNAs Encoding Fuc-TIII and Fuc-TV—Fuc-TIII-expressing plasmid pAmo-FT3 was selected from the SW1116 cDNA library by the same approach as that used in the cloning of Fuc-TVII. A Fuc-TVI-expressing vector pAmo-FT6 was obtained from the SW1116 cDNA library by the cloning approach with resistance selection against 10 μ g/ml *Maackia amurensis* lectin I, obtained from Vector Laboratories Inc., as described previously [18].

Isolation of cDNAs Encoding Fuc-TIV and Fuc-TV—A Fuc-TIV cDNA was amplified from single-stranded cDNAs of U-937 by polymerase chain reaction (PCR) using primers (5'-atcgaagcttGCAGCGCTGCCGTTCCGCCCAT-3' (synthetic HindIII site underlined) and 5'-atcgaagcttGCTTCACCGCTCGAACCAGCTGGC-3' (synthetic Asp⁷¹⁸ site underlined), digested with HindIII and Asp⁷¹⁸ and cloned between the HindIII and Asp⁷¹⁸ sites of pAmo [18], which generated a Fuc-TIV expressing plasmid pAmo-FT4. A pUC119-based plasmid carrying Fuc-TIV cDNA (named pUC119-FT4) was constructed by inserting into the HindIII site of pUC119 (41) a Fuc-TIV cDNA that was amplified from the single-stranded cDNAs of U-937 by PCR using primers (5'-GAGACGCTCAGGCCGCTGCTTTT-3' and 5'-GCAGGAGCCCAATTTCGGGCACT-3').

A Fuc-TV cDNA was amplified from human placenta chromosomal DNA (Clontech) by PCR using primers (5'-tcttaagcttCTCTCTTCCAGCTACTCTGACC-3' and 5'-gtcatagctcGCCGGCCTCTGGAACCAAGC-3'), digested with HindIII and SacI, and inserted between the HindIII and SacI sites of pUC119 to form pUC119-FT5.

PCR Analysis of Human Chromosomal Somatic Cell Hybrids—Chromosomal localization of the human Fuc-TVII gene was assigned by PCR analysis using genomic DNAs prepared from hamster-human somatic cell hybrids using both a kit (BIOSMAPTM Somatic Cell Hybrid PCRableTM DNAs, BIOS Co.) and the primers specific to the 3'-noncoding region of the Fuc-TVII gene (5'-TCAAACCACAGGCATCCC GCC-3' and 5'-CCTTCAACCCACCAAGATTGTTC-3'). The presence of the Fuc-TVII sequence was examined by 32 cycles of PCR of 94 °C for 30 s, 70 °C for 1 min, and 72 °C for 2 min. Under this condition, a predicted single 218-bp fragment was amplified from human genomic DNA but not from hamster DNA. To exclude the possibility of DNA contamination, control samples with no genomic DNA template were subjected to the same PCR analysis for each experiment. To confirm further that the amplified products originated from the Fuc-TVII gene they were subjected to SphI digestion, which divided the 218-bp Fuc-TVII fragment into 106- and 112-bp fragments.

Construction and Purification of Fuc-TVI and Fuc-TVII Protein Fused with Protein A—To analyze the Fuc-T activities of Fuc-TVI and Fuc-TVII, their respective putative catalytic domains were expressed as proteins fused with the IgG binding domain of *Staphylococcus aureus* protein A using the vector pAmoA as described [18]. A 0.97-kb fragment encoding a COOH-terminal portion of Fuc-TVI (amino acids 40-358) was amplified by PCR using primers 5'-ctctcgatattCCCACTGTACCTAATGGGTC-3' and 5'-gtagacggcgccCTCAGGTGAACCAACGCTATG-3', digested with EcoRV and NotI, and cloned between the StuI and NotI sites of pAmoA to yield pAmoA-FT6 expressing a protein A-Fuc-TVI fusion. A protein A-Fuc-TVII fusion (designated A6FT7) that contained a Fuc-TVI-derived peptide (amino acids 40-54) between protein A and Fuc-TVII (amino acids 39-342) portions was generated as follows. The 1.9-kb SacI fragment of pAmo-FT7 was inserted into the HindIII site of pUC119 to form pUC119-FT7. The 0.4-kb HindIII-Asp⁷¹⁸ fragment encoding both protein A and Fuc-TVI peptide was isolated from pAmoA-FT6 by PCR using primers 5'-CGCCAGTCCTCCGATGACTGAGT-3' and 5'-ccatggtaCCTGTGCTGCTGGGAAGCGGGA-3'. The 1.0-kb Asp⁷¹⁸-TaqI (blunt) fragment encoding a part of Fuc-TVI (amino acids 39-342) was excised from pUC119-FT7 and cloned into the HindIII and NotI (blunt) ends of pAmoA together with the 0.4-kb HindIII-Asp⁷¹⁸ fragment from pAmoA-FT6, which constructed pAmoA-FT7 expressing the protein A6FT7. The PCR-amplified portions of pAmoA-FT6 and pAmoA-FT7 were sequenced to confirm the absence of possible PCR errors.

Protein A, the protein A-Fuc-TVI fusion, and the fusion A6FT7 were expressed and purified from the conditioned medium of Namalwa KJM-1 cells transfected stably with plasmids pAmoA, pAmoA-FT6, or pAmoA-FT7, respectively, as described [18].

Fuc-T Assays and Product Characterization—These purified protein (5 μ l each) were assayed for Fuc-T activities in 0.1 M cacodylate buffer (pH 6.8), 5 mM ATP, 10 mM L-fucose, 75 μ M GDP-fucose (Wako Pure Chemicals), 25 mM MnCl₂, and 25 μ M pyridylaminated acceptor (in

Expression Cloning of a Novel α 1,3-Fucosyltransferase That Is Involved in Biosynthesis of the Sialyl Lewis x Carbohydrate Determinants in Leukocytes*

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The sialyl Lewis x (NeuAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc) determinants serve as ligands in the selectin-mediated adhesion of leukocytes to activated endothelium or platelets. In our efforts to identify glycosyltransferases involved in the biosynthesis of those ligands, we achieved expression cloning of a novel human α 1,3-fucosyltransferase termed Fuc-TVII from a THP-1 cDNA library by enrichment of the Namalwa cells highly expressing that determinant with a fluorescence-activated cell sorter. Expression of the COOH-terminal catalytic domain of Fuc-TVII showed an α 1,3-fucosyltransferase activity for a type II oligosaccharide with a terminal α 2,3-linked sialic acid among various acceptors, consistent with that *in vivo* acceptor specificity. Alignment of the primary sequences of five α 1,3-fucosyltransferases and assignment of the chromosomal location of Fuc-TVII gene, together with that acceptor specificity, indicate that Fuc-TVII consists of a unique class of the α 1,3-fucosyltransferase family. Determination of the expression levels of these α 1,3-fucosyltransferases in various cells revealed that both Fuc-TVII and a myeloid fucosyltransferase Fuc-TIV were significantly expressed in myeloid lineage cells. Fuc-TVII-transfected Namalwa cells exhibited significant binding to E-selectin in contrast to little binding of the Fuc-TIV-transfected cells. These results suggest that Fuc-TVII may participate in the biosynthesis of the selectin ligands.

Much evidence has accumulated indicating that sialylated and/or fucosylated lactosaminoglycans are involved in fetal development, tumorigenesis, hematopoietic cell differentiation, and leukocyte trafficking (1). In particular, the sialyl Lewis x (sLe^x) (NeuAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc) determinant

and its stereoisomer sialyl Lewis a (sLe^a) (NeuAc α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc) determinant have evoked considerable interest because both were demonstrated to serve as ligands for the three known selectins (E-, P-, and L-selectins) (2-11), which are cell adhesion molecules involved in the recruitment of leukocytes into lymphoid tissues and sites of inflammation (12, 13). Furthermore, these determinants and their related structures also have been reported to be associated with cancer malignancy (14-17), and a number of monoclonal antibodies (mAbs) against those antigens have been generated for cancer diagnosis (16, 17).

The mechanisms for regulating the biosynthesis of these determinants in various cells are of critical importance for understanding their roles in the above biological processes. Their biosynthesis will be controlled largely by glycosyltransferases, especially α 2,3-sialyltransferases and α 1,3- and α 1,4-fucosyltransferases (Fuc-Ts). The identification of glycosyltransferases involved in the respective biological processes will therefore help to explain such mechanisms at the molecular level. To isolate the glycosyltransferases participating in the biosynthesis of the sLe^x and sLe^a determinants, we have developed a novel cloning approach using lectin resistance selection and reported expression cloning of a novel α 2,3-sialyltransferase (termed ST-4), which directed *de novo* expression of the sLe^a determinant (18).

To date, four α 1,3-Fuc-Ts (designated Fuc-TIII, Fuc-TIV, Fuc-TV, and Fuc-TVI) have been cloned and characterized (4, 20-26). Except Fuc-TIV, these enzymes were reported to direct the appearance of the sLe^x and/or sLe^a determinants *in vivo* (4, 22-26). However, it is still unclear which Fuc-T is responsible for the biosynthesis of the selectin carbohydrate ligands, since the expression patterns of these Fuc-Ts, except Fuc-TIV, have not been analyzed in detail. Goetz *et al.* (21) showed that ELAM-1 ligand fucosyltransferase, identical to Fuc-TIV, was expressed in a myeloid lineage cell line HL-60 and conferred E-selectin binding activity to transfected Chinese hamster ovary (CHO) cells. However, the capability of Fuc-TIV of generating the selectin ligands in either transfected CHO and COS cells has not been confirmed by other studies (22, 23). As a possible explanation for this discrepancy, the involvement of host-specific factors such as specific glycoproteins or glycolipids presenting the selectin carbohydrate ligands has been postulated (22). In this light, it will be of crucial importance to examine whether Fuc-TIV can direct increased expression of the sLe^x determinant in hosts of leukocyte origin, which have the potential of binding to activated endothelial cells. An alternative hypothesis for the biosynthesis of the selectin ligands is

lacto-N-fucopentaose V (Gal β 1-3GlcNAc α 1-3Gal β 1-4[Fuc α 1-3]GlcNAc), high performance liquid chromatography; FACS, fluorescence-activated cell sorter

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† The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession numbers: X78031.

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The abbreviations used are: sLe^x, sialyl Lewis x (NeuAc α 2-3Gal β 1-4[Fuc α 1-3]GlcNAc); sLe^a, sialyl Lewis a (NeuAc α 2-3Gal β 1-3[Fuc α 1-4]GlcNAc); mAb, monoclonal antibody; Fuc-T, fucosyltransferase; CHO, Chinese hamster ovary; PMN, polymorphonuclear leukocytes; kb, kilobases; Le^x, Lewis x (Gal β 1-4[Fuc α 1-2]GlcNAc); O₆, H⁺NeuAcGlcNAc₆; Cer, Lewis a (Gal β 1-3[Fuc α 1-4]GlcNAc); PCR, polymerase chain reaction; bp, base pairs; LNT, lacto-N-neotetraose (Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc); LNT, lacto-N-tetraose (Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc); LNFP-I, lacto-N-fucopentaose I (Fuc α 1-2Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc); LNFP-II, lacto-N-fucopentaose II (Gal β 1-3[Fuc α 1-4]GlcNAc β 1-3Gal β 1-4Glc); LNFP-III, lacto-N-fucopentaose III (Gal β 1-4[Fuc α 1-3]GlcNAc β 1-3Gal β 1-4Glc); LNFP-V,

D8

Specificity Analysis of Three Clonal and Five Non-Clonal α 1,3-L-Fucosyltransferases with Sulfated, Sialylated, or Fucosylated Synthetic Carbohydrates as Acceptors in Relation to the Assembly of 3'-Sialyl-6'-sulfo Lewis x (the L-Selectin Ligand) and Related Complex Structures[†]

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ABSTRACT: Unique specificities of the cloned α 1,3-L-fucosyltransferases (FTs), FT III (Lewis type), FT IV (myeloid type), and FT V (plasma type), and the α 1,3-FTs of Colo 205 (colon carcinoma), HL 60 (myeloid), B142 (lymphoid), EKVX (lung carcinoma), and calf mesenteric lymph nodes (CMLN) were discerned with sulfated, sialylated, and/or fucosylated Gal β 1,3/4GlcNAc β -based acceptor moieties. (a) FT V was 1.0-, 20.8-, and 4.6-fold active in forming Lewis x, Lewis y, and 3'- α -galactosyl Lewis x, respectively. (b) FT III and FT V formed \sim 4-fold 3'-sulfo Lewis x, as compared to 3'-sialyl Lewis x. (c) FT IV showed great efficiency in forming 3'-sulfo Lewis x (249%) and Lewis x (345%) in mucin-type branched chains. (d) FT III, FT IV, and FT V formed 19%, 62%, and 47% 6-sulfo Lewis x as compared to Lewis x. (e) 6'-Sulfo Lewis x and 3'-sialyl-6'-sulfo Lewis x (GLYCAM ligand) were not synthesized from their immediate precursors by FT III, FT IV, or FT V. (f) FT III, FT IV, and FT V were 311%, 9%, and 188% active, respectively, with 2'-fucosyl lactose but were not active with 2'-fucosyl-6'-sulfo lactose. (g) FT III and FT V were 7.0- and 0.5-fold active in forming Lewis a as compared to Lewis x, whereas, FT IV was inactive. (h) FT III was 2.0-fold more active in forming 3'- α -galactosyl Lewis a than Lewis b. (i) FT III synthesized 6-sialyl Lewis a (40% efficiency as compared to Lewis a) from 6-sialyl type 1. (j) FT III did not act on 6'-sulfo or 6'-sialyl type 1 but was 106% and 22% active with 3'-sulfo and 6-sulfo type 1, respectively. (k) The Colo 205 FT activities with type 1 compounds almost paralleled that of FT III except for the low activity (9%) with Gal β 1,3(NeuAc α 2, 6)GlcNAc β -O-Bn, but with type 2 considerable differences between Colo 205 FT and FT III were noticed. (l) The α 1,3-FTs of CMLN, HL60, B142, and EKVX were 1.2–1.7 times active with Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP and Gal α 1,3Gal β 1,4GlcNAc β -O-Bn with respect to Gal β 1,4GlcNAc β -O-Al. (m) Both CMLN and HL60 FTs were 2-fold active with 3-sulfoGal β 1,4GlcNAc in a mucin-type branch structure such as 3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn. (n) The 3'-sulfoLacNAc/acrylamide copolymer, either as an acceptor or as a competitive inhibitor, had the potential to distinguish myeloid type α 1,3-FT from the plasma type.

The ligands for E- and P-selectins, two members of the selectin family of cell adhesion molecules, have been characterized as sialylated and fucosylated oligosaccharides (Lowe et al., 1990; Phillips et al., 1990; Walz et al., 1990; Polley et al., 1991; Berg et al., 1991; Takada et al., 1991). The ligand for the third member, L-selectin, has been shown to constitute a sulfate group in addition to sialic acid and fucose (Imai et al., 1991). In addition, the sulfated Lewis a tetra and pentasaccharides of ovarian cystadenocarcinoma glycoprotein were shown to be potent E-selectin ligands (Yuen et al., 1994). Presently, it is becoming evident that subtle differences in the structure of sialylated, fucosylated oligosaccharides influence their binding affinity with E- and

L-selectins (Foxall et al., 1992; Needham & Schnaar, 1993). The expression of fucosylated oligosaccharides is largely controlled by regulating the expression of α 1,3-fucosyltransferases (FTs)¹ (Lowe, 1991). Recent studies have led to the recognition of five different human α 1,3-FTs, designated Fuc-T III to Fuc-T VII. Fuc-T III corresponds to the Lewis type α 1,3/4-FT (Kukowska-Latallo et al., 1990), Fuc-T IV corresponds to the myeloid type (Goelz et al., 1990; Lowe et al., 1991; Kumar et al., 1991), Fuc-T V and Fuc-T VI correspond to the plasma type (Weston et al., 1992a,b; Koszdin & Bowen, 1992), and Fuc-T VII appears to be a unique type (Sasaki et al., 1994). Yago et al. (1993) examined the expression of Fuc-T III to Fuc-T VI at mRNA levels in various epithelial cancer and leukemia cell lines and found mixtures of multiple molecular species of these FTs. Recently, Sasaki et al. (1994) studied the expression level of the five cloned α 1,3-FTs and found both Fuc-T IV

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¹ Abbreviations: FT, fucosyltransferase; Al, allyl; Bn, benzyl; Me, methyl; pNP, *para*-nitrophenyl; AA-CP, acrylamide copolymer; CMLN, calf mesenteric lymph nodes; TLC, thin-layer chromatography.

and Fuc-T VII in myeloid lineage cells. When expressed in the human Burkitt lymphoma cell line, Namalwa, both of these enzymes formed sialyl Lewis x at the cell surface, but the E-selectin binding ability was only conferred on these cells by Fuc-T VII. Sueyoshi et al. (1994) compared Chinese hamster ovary (CHO) cells, stably transfected with Fuc-T III (CHO-FT III) or Fuc-T IV (CHO-FT IV), for their carbohydrate structures and for their binding to E- or L-selectin. CHO-FT III expressed sialyl Lewis x, Lewis x, and VIM-2 structures, whereas CHO-FT IV expressed only Lewis x and a small amount of VIM-2. They also found that only CHO-FT III adhered to E-selectin and that both CHO-FT III and CHO-FT IV failed to adhere to L-selectin. The present paper reports our detailed investigation on the intricate specificities of the cloned Fuc-T III, Fuc-T IV, and Fuc-T V and also on the specificities of α 1,3-FTs present in some human cell lines and calf lymph node using a variety of sulfated, sialylated, and fucosylated compounds as acceptors. The present study has led to several unique findings on the specificities of these enzymes, thus forming a sound basis to explain their distinct roles in the expression of biological carbohydrate ligands and carbohydrate antigenic determinants.

EXPERIMENTAL PROCEDURES

FT III, FT IV, and FT V. These cloned and expressed enzyme-Protein A fusion products (Glycomed, Alameda, CA) were isolated by binding to IgG-Sepharose beads (1 mL of IgG-Sepharose/L of the condition media containing the protein A-enzyme), followed by dissociation of that complex for soluble enzyme. As the present studies necessitated soluble enzymes, 1.0 mL of the Sepharose bead slurry of each FT III, FT IV, and FT V was centrifuged for 1 min in a microfuge. After removal of the supernatant, 1.0 mL of 1 mM Tris-HCl-150 mM NaCl, pH 8.0, was added to the beads, mixed gently by finger tapping, and centrifuged. The supernatant was discarded, and then 1.0 mL of 0.1 M citrate buffer, pH 4.4, was added to the beads, mixed in the cold room for $\frac{1}{2}$ h using Speci-Mix (Thermolyne), and centrifuged for 1 min. The supernatant was mixed with 1.0 mL of 0.5 M Hepes, pH 7.5, containing 4% Triton X-100 and 20 mg of BSA and then dialyzed overnight at 4 °C against 1 L of 25 mM Tris-HCl, pH 7.0, containing 35 mM MgCl₂, 1 mM ATP, and 10 mM NaN₃. The enzyme solutions were stored at 4 °C, and there was no appreciable loss of enzyme activity for at least 2 months. In each assay, 5 μ L of the soluble enzyme preparation was used.

Cell Culture. Colo 205, HL60, B142, and EKVX were grown in 250 mL plastic T-flasks in RPMI 1640 as described earlier (Chandrasekaran, 1995a,b). The cells were homogenized with Tris-buffered saline, pH 7.0, containing 2% Triton X-100 using a Dounce all-glass hand-operated grinder. The homogenate was centrifuged at 20 000g for 1 h at 4 °C. The supernatant was adjusted to 1 mg of protein/mL by adding the necessary volume of the extraction buffer. 5 μ L aliquots of these extracts were used in assays run in duplicate. Protein was measured by the BCA method (Pierce Chemical Co.) with BSA as the standard. Calf mesenteric lymph node was made available from the animal facility in Springville, NY, through the courtesy of Dr. M. P. McGarry.

Human breast tumor and human ovarian tumor were obtained from the tissue facility of Roswell Park Cancer

Institute. All tissue specimens were kept frozen at until use.

These tissues were homogenized using Kinematic buffered saline and then stirred for 1 h at 4 °C after the concentration of Triton X-100 to 2%. Homogenate was centrifuged at 20 000g for 1 h at 4 °C. The clear supernatant was adjusted to 10 mg of protein/mL by the necessary volume of Tris-buffered saline-2% Triton X-100. 5 μ L aliquots were used in the assay.

Assay of α 1,3- and α 1,4-FT Activities. The incubation mixtures run in duplicate contained 50 mM Hepes, pH 7.5, 5 mM MnCl₂, 7 mM ATP, 3 mM NaN₃, the acceptor (3.0 mM unless otherwise stated), 0.05% μ Ci of [¹⁴C]Fuc (specific activity 290 mCi/mmol), and 5 μ L of enzyme solution in a total volume of 20 μ L. Incubation mixtures had everything except the acceptor; at the end of incubation for 4 h at 37 °C the mixture was added with 1.0 mL of water and passed through a Dowex column (1 mL in a Pasteur pipet) (Chandrasekaran, 1992). The column was washed twice with 1 mL of water. The breakthrough and wash, which contained the fucosylated neutral acceptor, were collected together in a scintillation vial, and radioactive content was determined using the 3a70 scintillation mixture (Research Products International, Mount Prospect, IL) and a Beckman LS 5000TD instrument. The Dowex column was then eluted with 1 mL of 0.2 M NaCl to obtain the [¹⁴C]fucosylated product from sialylated/sulfated acceptors and then counted for radioactivity as above. Corrections were made by subtracting the radioactivity in the water and NaCl eluates of the incubation mixtures from the values of the corresponding eluates of the tests. Values for the duplicate runs could vary more than 5%.

Synthetic Compounds. We already reported the synthesis of many of these compounds used in the present study (Chandrasekaran et al., 1993a, 1994; Chandrasekaran et al., 1995). Details on the remaining compounds are reported elsewhere.

Acrylamide-Sulfoglycan Copolymers. Acrylamide polymers of 3-sulfoGal β 1,3GlcNAc β -O-Al and 3-sulfoGal β 1,4GlcNAc β -O-Al were synthesized by following the procedure of Horejsi et al. (1978). About 1.0 μ mol of sugar unit was present in 1.0 mg of these copolymers (determination of Gal by anthrone reaction); these copolymers exhibited an approximate molecular weight of 40 000 as judged by chromatography on a Bio-Gel P60 column with dextran of 39 200 average molecular weight as the marker.

Acceptor Competition Experiments with Copolymers

(A) Competition between the acceptors and acrylamide-sulfoglycan copolymers for Colo 205 α 1,3/4-FTs. The effect of 3-sulfoGal β 1,3GlcNAc β -O-Al/AA-CP on the α 1,3-FT as well as α 1,4-FT activities of Colo 205 was measured using the Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP and Fuc α 1,2Gal β 1,3GlcNAc β -O-pNP acceptors, respectively. The effect of 3-sulfoGal β 1,4GlcNAc β -O-Al/AA-CP on the α 1,3-FT as well as the α 1,4-FT activities of Colo 205 was measured using the Gal β 1,4GlcNAc β -O-Al and Gal β 1,3GlcNAc β -O-Al acceptors, respectively. The concentration of copolymer in the reaction mixture varied between 2.5 and 100.0 μ M (based on a molecular weight of 40 000) under the standard incubation conditions. The transfer of [¹⁴C]Fuc to the neutral acceptors was measured by the Dowex method against

Table 1: Differentiation of the Specificities of Colo 205 and the Cloned α 1,3-L-Fucosyltransferases (FT III, FT IV, and FT V) with Type 2 (Gal β 1,4GlcNAc β -)-Based Synthetic Carbohydrates as Acceptors

synthetic carbohydrate (3.0 mM)	fucosyltransferase activity, incorporation of [14 C]Fuc (CPM $\times 10^{-3}$)			
	Colo 205	FT III	FT IV	FT V
Gal β 1,4GlcNAc β -O-Al	26.95 (100)	2.88 (100)	3.75 (100)	0.62 (100)
Fuc α 1,2Gal β 1,4GlcNAc β -O-PNP	27.12 (101)	12.16 (423)	17.53 (470)	12.80 (2075)
Fuc α 1,2Gal β 1,4Glc	16.45 (61)	8.93 (311)	0.32 (9)	1.16 (188)
Gal α 1,3Gal β 1,4GlcNAc β -O-Bn	29.11 (108)	2.66 (92)	6.45 (172)	2.83 (458)
NeuAc α 2,3Gal β 1,4GlcNAc β -O-Bn	15.60 (58)	1.31 (46)	0.23 (6)	0.54 (88)
3-sulfoGal β 1,4GlcNAc	22.37 (83)	4.42 (154)	1.41 (37)	2.13 (345)
Gal β 1,4(6-sulfo)GlcNAc β -O-Al	13.02 (48)	0.55 (19)	2.32 (62)	0.29 (47)
6-sulfoGal β 1,4GlcNAc	1.84 (7)	0.06 (2)	0 (0)	0.01 (1)
6-sulfoGal β 1,4(3-O-Me)	0.05 (<1)	0.02 (<1)	0 (0)	0.01 (1)
GlcNAc β -O-Bn				
6-sulfoGal β 1,4GlcNAc β -O-Me	0 (0)	0.02 (<1)	0.05 (1)	0.02 (3)
NeuAc α 2,3(6-sulfo)Gal β 1,4GlcNAc β -O-Me	0 (0)	0.04 (1)	0 (0)	0.02 (3)
Fuc α 1,2(6-sulfo)Gal β 1,4Glc	0.03 (<1)	0.01 (<1)	0.01 (<1)	0.01 (1)
3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn	15.02 (56)	2.98 (104)	9.33 (249)	2.21 (358)
Gal β 1,4GlcNAc β 1,6(3-sulfoGal β 1,3)GalNAc α -O-Bn	16.19 (60)	0.54 (19)	12.94 (345)	0.34 (55)

K_i for the inhibition of [14 C]Fuc transfer to the neutral acceptor by the acrylamide sulfoglycan copolymer was determined by Lineweaver–Burke plot.

(B) Competition between the acceptors and acrylamide–sulfoglycan copolymers for HL60 α 1,3-FT and the cloned FT III (α 1,4-FT): The effects of both 3-sulfoGal β 1,3GlcNAc β -O-Al/AA-CP and 3-sulfoGal β 1,4GlcNAc β -O-Al/AA-CP on the α 1,3-FT activity of HL60 and on the α 1,4-FT activity of FT III were measured using Gal β 1,4GlcNAc β -O-Al and Gal β 1,3GlcNAc β -O-Al as the respective acceptors under standard incubation conditions in the presence of the copolymer as described above. K_i for the inhibition was calculated by Lineweaver–Burke plot.

RESULTS AND DISCUSSION

Type 2 (Gal β 1,4GlcNAc β -)-Based Structures as the Acceptors for Cloned Enzymes FT III, FT IV, and FT V. See Table 1.) Blood group H type 2 [Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP] served as the most efficient acceptor. As compared to Gal β 1,4GlcNAc β -O-Al, enzyme activity with this acceptor was about 4-fold more with both FT III and FT IV. It is highly interesting to note that FT V showed more than 20-fold activity with Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP as compared to the basic type 2 (Gal β 1,4GlcNAc β -O-Al). FT III and FT V showed 311% and 188% activity with 2'-fucosyllactose as compared to Gal β 1,4GlcNAc β -O-Al, whereas FT IV exhibited very low activity (9%). If the α 1,2-fucosyl group in 2'-fucosylLacNAc β - is replaced by an α 1,3-linked Gal, FT III activity was reduced from 423% to 92%, FT IV activity was reduced from 470% to 172%, and FT V activity was reduced from 2075% to 458%. These results imply that even though these enzymes prefer H type 2 as the acceptor, they are also capable of synthesizing 3'- α -galactosyl Lewis x from 3'- α -galactosylLacNAc at a significant level. When a sialyl group is linked α 2,3 to Gal in Gal β 1,4GlcNAc β -, FT III and FT V showed 46% and 88% activity, respectively, whereas the activity of FT IV was almost negligible (only 6%). 3'-SulfoLacNAc was highly reactive with FT III (154%) and FT V (345%) and showed less activity with FT IV (37%).

FT IV was the only enzyme that showed appreciably more activity with Gal β 1,4(6-sulfo)GlcNAc β -O-Al (62%) as compared to 3-sulfoGal β 1,4GlcNAc (37%), while FT III and FT V showed much lower activities (19% versus 154% and 47%

versus 345%, respectively). When sulfate was present on C-6 of Gal, the acceptor activity was lost toward all of these enzymes [see the activities with the following acceptors: 6-sulfoGal β 1,4GlcNAc; 6-sulfoGal β 1,4(3-O-Me)GlcNAc β -O-Bn; 6-sulfoGal β 1,4GlcNAc β -O-Me; NeuAc α 2,3(6-sulfo)-Gal β 1,4GlcNAc β -O-Me; and Fuc α 1,2(6-sulfo)Gal β 1,4Glc].

A very interesting observation was made on FT IV when its activity with an acceptor containing either Gal β 1,4GlcNAc or 3-sulfoGal β 1,4GlcNAc linked β 1,6 to GalNAc of the T-hapten was examined. FT IV showed 249% activity with 3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn as compared to 37% activity with 3-sulfoGal β 1,4GlcNAc-, whereas FT V showed almost the same activity (358% and 345%, respectively) and FT III exhibited significantly less activity (104% and 154%, respectively). When Gal β 1,4GlcNAc β 1,6-(3-sulfoGal β 1,3)GalNAc α -O-Bn was used as an acceptor, FT III and FT V showed much lower activity (19% and 55%, respectively), whereas FT IV was 345% active. Such a high activity exhibited by FT IV and not by FT III and FT V toward this acceptor suggests that the chain termination on the β 1,3 branch has no effect on the activity of FT IV toward the β 1,6 branch. It is reasonable to state that the branched structures noted above are preferred acceptors for FT IV.

Type 1 (Gal β 1,3GlcNAc β -)-Based Structures as Acceptors for Cloned Enzymes FT III, FT IV, and FT V. The acceptors containing type 1, namely, Gal β 1,3GlcNAc β -O-Al, Fuc α 1,2Gal β 1,3GlcNAc β -O-pNP, and Gal α 1,3Gal β 1,3GlcNAc β -O-Bn, were almost inactive with FT IV. FT V showed low activity with these acceptors (see Table 2) as compared to its activity with 2'-fucosylLacNAc β -O-pNP (Table 1). On the contrary, FT III was quite active with type 1 acceptors. As compared to Gal β 1,4GlcNAc β -O-Al, Gal β 1,3GlcNAc β -O-Al was 7-fold active (CPM $\times 10^{-3}$ = 2.88 versus 20.91). H type 1 as well as the 3'-sulfo derivative of type 1 had the same degree of activity (98% and 106%, respectively), whereas Gal α 1,3Gal β 1,3GlcNAc β -O-Bn exhibited 186% activity.

Substitution on C-6 of GlcNAc with a sulfo group in type 1 chain reduced the activity of FT III to 22%, whereas substitution in the same position with a sialyl group, surprisingly, retained 40% acceptor activity. Substitution with O-methyl retained 100% activity. Type 1 acceptors containing either the 6-sulfo or 6-sialyl group on Gal exhibited negligible amounts of activity [see the following

Table 2: Reactivity of Colo 205 and the Cloned α 1,3-L-Fucosyltransferases (FT III, FT IV, and FT V) with Type 1 (Gal β 1,3GlcNAc β -)-Based Synthetic Carbohydrates

synthetic carbohydrate (3.0 mM)	fucosyltransferase activity, incorporation of [14 C]Fuc (CPM $\times 10^{-3}$)			
	Colo 205	FT III	FT IV	FT V
Gal β 1,4GlcNAc β -O-Al	26.95	2.88	3.75	0.62
Gal β 1,3GlcNAc β -O-Al	27.10 (100)	20.91 (100)	0.04 (100)	0.32 (100)
Fuc α 1,2Gal β 1,3GlcNAc β -O-pNP	27.49 (101)	20.47 (98)	0.30 (350)	0.31 (97)
Gal α 1,3Gal β 1,3GlcNAc β -O-Bn		38.84 (186)	0.05 (125)	0.69 (216)
Gal β 1,3(Fuc α 1,4)GlcNAc β -O-Al	3.38 (12)	1.53 (7)		
Gal β 1,3(4-O-Me)GlcNAc β -O-Bn	5.30 (20)	2.64 (13)		
Gal β 1,3(6-O-Me)GlcNAc β -O-Bn	29.98 (111)	20.85 (100)		
Gal β 1,3(4,6-di-O-Me)GlcNAc β -O-Bn	1.88 (7)	0.77 (4)		
Gal β 1,3(NeuAc α 2,6)GlcNAc β -O-Bn	2.52 (9)	8.30 (40)		
NeuAc α 2,6Gal β 1,3GlcNAc β -O-Bn	0.18 (<1)	0.06 (<1)		
6-sulfoGal β 1,3GlcNAc β -O-Al	2.04 (8)	1.02 (5)		
Gal β 1,3(6-sulfo)GlcNAc β -O-Bn	8.85 (33)	4.53 (22)		
3-sulfoGal β 1,3GlcNAc β -O-Al	21.98 (81)	22.23 (106)		
3-sulfoGal β 1,3(6-O-Me)GlcNAc β -O-Al	17.65 (65)	18.13 (87)		
Fuc α 1,2(6-sulfo)Gal β 1,3GlcNAc β -O-Al	0 (0)	0.02 (<1)		

Table 3: Discerning the Specificities of α 1,3-Fucosyltransferases Present in Calf Mesenteric Lymph Node (CMLN) and HL60 (Myeloid), B142 (Lymphoid), and EKVX (Lung Adenocarcinoma) Cell Lines

synthetic carbohydrate (3.0 mM)	fucosyltransferase activity, incorporation of [14 C]Fuc (CPM $\times 10^{-3}$)			
	CMLN	HL60	B142	EKVX
Gal β -O-Bn	0 (0)	0.15 (<1)	0.03 (<1)	0.08 (<1)
Gal β 1,4GlcNAc β -O-Al	27.15 (100)	26.81 (100)	5.54 (100)	19.37 (100)
Gal β 1,3GlcNAc β -O-Al	0.20 (<1)	0.20 (<1)	0 (0)	0 (0)
Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP	31.22 (115)	37.80 (141)	9.20 (166)	30.41 (157)
Fuc α 1,2Gal β 1,4GlcNAc β -O-Bn	1.59 (6)	2.70 (10)		0.39 (2)
Gal α 1,3Gal β 1,4GlcNAc β -O-Bn	31.20 (115)	35.70 (133)	8.81 (159)	30.40 (157)
NeuAc α 2,3Gal β 1,4GlcNAc β -O-Bn	2.43 (9)	3.46 (13)	0.66 (12)	1.36 (7)
3-sulfoGal β 1,4GlcNAc β -O-Bn	8.53 (31)	9.80 (37)	1.44 (26)	4.46 (23)
6-sulfoGal β 1,4GlcNAc β -O-Me	0.83 (3)	2.02 (8)		0.16 (<1)
NeuAc α 2,3(6-sulfo)Gal β 1,4GlcNAc β -O-Me	0.01 (<1)	0.36 (1)		0 (0)
Fuc α 1,2(6-sulfo)Gal β 1,4GlcNAc β -O-Bn	0 (0)	0 (0)		
3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn	18.62 (69)	20.68 (77)		
Gal β 1,4GlcNAc β 1,6(3-sulfoGal β 1,3)GalNAc α -O-Bn	16.42 (60)	16.53 (62)		

acceptors: 6-sulfoGal β 1,3GlcNAc β -O-Al; NeuAc α 2,6Gal β 1,3GlcNAc β -O-Bn; and Fuc α 1,2(6-sulfo)Gal β 1,3GlcNAc β -O-Al].

FT V exhibited (see Table 2) the activities of 100%, 194%, and 97%, respectively, toward Gal β 1,3GlcNAc β -O-Al, Gal β 1,4GlcNAc β -O-Al, and Fuc α 1,2Gal β 1,3GlcNAc β -O-pNP. The results thus lend support to the suggestion of Henry et al. (1995) that FT V is a good candidate for the production of some Lewis antigen by Lewis negative individuals.

Activity of Colo 205 α 1,3/4-FT toward Type 1 and Type 2 Structures. In Colo 205 α 1,3- and α 1,4-FT activities (refer Table 2) toward their respective acceptors, Gal β 1,4GlcNAc β -O-Al and Gal β 1,3GlcNAc β -O-Al, were almost equal, whereas the α 1,3-FT activity was less than 15% of the α 1,4-FT activity in FT III. The Colo 205 FT activities (expressed as % of the activity with Gal β 1,3GlcNAc β -O-Al) with various type 1 acceptors almost paralleled that of FT III except for the acceptor Gal β 1,3(NeuAc α 2,6)GlcNAc β -O-Bn, which was less active with Colo 205 FT (9%). Considerable differences, however, were noticed in Colo 205 FT and FT III activity toward type 2 containing structures. H type 2 exhibited the same activity as the basic type 2 (Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP, 101%) with Colo 205 FT, whereas FT III exhibited ~4-fold activity with H type 2. On the contrary, while Gal α 1,3Gal β 1,4GlcNAc β -O-Bn and NeuAc α 2,3Gal β 1,3GlcNAc β -O-Bn respectively exhibited less than 1/4 and 1/5 activity with FT III, their activities with

Colo 205 FT were 108% and 58%, respectively, when compared to that toward Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP.

Also, compared to Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP, 3-sulfoGal β 1,4GlcNAc β and Gal β 1,4(6-sulfo)GlcNAc β -O-Bn respectively showed 83% and 48% activity with Colo 205 FT, whereas with FT III their respective activities were only 35% and 5%. Type 2 acceptors containing 6-sulfated Gal β also showed negligible activity with Colo 205 FT.

As observed with FT III, the 3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn acceptor was less active than 3-sulfoGal β 1,4GlcNAc β with Colo 205 FT (56% and 83% respectively). In contrast to FT III which was only 1/5 as active with Gal β 1,4GlcNAc β 1,6(3-sulfoGal β 1,3)GalNAc α -O-Bn when compared to its activity with 3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn, the activity of Colo 205 FT with these acceptors was almost equal.

Activities of α 1,3-FT Present in Calf Mesenteric Lymph Nodes (CMLN), HL60 (Myeloid), B142 (Lymphoid), and EKVX (Lung Adenocarcinoma). The above sources (see Table 3) showed either 0% or <1% activity with the acceptors Gal β -O-Bn and Gal β 1,3GlcNAc β -O-Al, indicating that α 1,2- and α 1,4-FT activities are almost non-existent in these sources. Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP and Gal α 1,3Gal β 1,4GlcNAc β -O-Bn were better acceptors for α 1,3-FT in these sources as compared to Gal β 1,4GlcNAc β -O-Al. 2'-Fucosyllactose and 3'-sialyllactose exhibited low acceptor activity with these sources. Considerable

Table 4: Differentiation of α 1,3-L-Fucosyltransferases on the Basis of Differences in Their Affinities toward 2'-FucosylLacNAc β -O-pNP and 3'-SulfoLacNAc β -O-A/AA-CP

enzyme source	α 1,3-L-fucosyltransferase activity, incorporation of [14 C]Fuc into the acceptor (CPM $\times 10^{-3}$ /mg of protein)		
	A, 2'-fucosylLacNAc β -O-pNP ^a (3.0 mM)	B, 3'-sulfoLacNAc β -O-A/AA-CP ^b (62.5 μ M)	ratio, B/A (%)
Colo 205 (colon carcinoma)	663.2	392.9	59.2
HL60 (myeloid)	487.5	43.5	8.9
B142 (lymphoid)	85.3	3.4	4.0
calf mesenteric lymph node	631.0	124.9	19.8
human breast tumor	122.2	9.0	7.4
human ovarian tumor	91.6	10.0	10.9

^a The radioactive product from this acceptor was measured by Dowex-I-Cl method. ^b The radioactive product from this acceptor remained at the origin of silica gel GHLF plates after chromatography using ethylacetate:pyridine:water:acetic acid (5/5/3/1). This was quantitated by scraping the silica gel into scintillation vials containing 2.0 mL of water and then liquid scintillation counting. Correction was made by subtracting the radioactivity at the origin of TLC plates from the blanks containing no acceptor.

Table 5: Effect of Copolymers on the α 1,3/4-FT Activities

copolymer	enzyme source	Inhibition of FT Activity (%)	
		α 1,3	α 1,4
3-sulfoGal β 1,3GlcNAc β -O-A/AA-CP (62.5 μ M)	Colo 205	56.1	90.4
	cloned FT III (Lewis type)	ND ^a	84.3
3-sulfoGal β 1,4GlcNAc β -O-A/AA-CP (62.5 μ M)	HL60	0	0
	Colo 205	54.6	0
	cloned FT III (Lewis type)	ND	0
	HL60	0	0

^a ND, not determined.

activity (23%–37%) was observed with 3'-sulfoLacNAc. CMLN and HL60 FTs were active to a very small extent with 6'-sulfoLacNAc β -O-Me (3% and 8%, respectively), whereas no activity was observable with 3'-sialyl-6'-sulfoLacNAc β -O-Me, and 2'-fucosyl-6'-sulfolactose.

Both CMLN and HL60 showed more than 2-fold activity with 3-sulfoGal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn as compared to 3-sulfoGal β 1,4GlcNAc. As compared to their activities with Gal β 1,4GlcNAc β 1,6(Gal β 1,3)GalNAc α -O-Bn, CMLN and HL60 also showed an almost equal amount of activity with Gal β 1,4GlcNAc β 1,6(3-sulfoGal β 1,3)GalNAc α -O-Bn, indicating that the termination of the β 1,3 branch does not inhibit the activity of α 1,3-FTs of CMLN and HL 60 on the other branch.

Acceptor 3'-SulfoLacNAc β -O-A/AA-CP in Conjunction with 2'-FucosylLacNAc β -O-pNP Serves as a Tool in Differentiation of α 1,3-L-Fucosyltransferases. (See Table 4.) A substantial difference was noticed in each case between the α 1,3-L-FT activities measured by 2'-fucosylLacNAc β -O-pNP and the copolymer. When the ratio of the activities measured by the two acceptors was calculated for each case and the resulting values were compared, it was found that the α 1,3-FT of Colo 205 differs markedly from the other α 1,3-FTs. Further, the α 1,3-FT of calf mesenteric lymph node also differed substantially from the α 1,3-FTs of HL 60, B142, and breast and ovarian tumors.

Effect of Acrylamide Copolymers Containing either 3-SulfoGal β 1,3GlcNAc β - or 3-SulfoGal β 1,4GlcNAc β - Units on α 1,3-Fucosyltransferase Activities. (See Table 5.) When α 1,3- and α 1,4-FT activities of Colo 205 were measured in the presence of increasing concentrations of 3-sulfoGal β 1,4GlcNAc β -O-A/AA-CP, using the Gal β 1,4GlcNAc β -O-Al and Gal β 1,3GlcNAc β -O-Al acceptors (see Figure 1), inhibition of the α 1,3-FT activity and not the α 1,4-FT activity was noticed; a maximum inhibition of about 50%

was reached at the minimum concentration of 25 μ M of the copolymer. When α 1,3- and α 1,4-FT activities of Colo 205 were measured in the presence of increasing concentration of 3-sulfoGal β 1,3GlcNAc β -O-A/AA-CP, using the Fuc α 1,2Gal β 1,4GlcNAc β -O-pNP and Fuc α 1,2Gal β 1,3GlcNAc β -O-pNP acceptors, inhibition of both α 1,3- and α 1,4-FT activities were seen. At 25 μ M of this copolymer, ~50% inhibition (the maximum attainable) of this α 1,3-FT activity and 75% inhibition of the α 1,4-FT activity were reached; a maximum inhibition (90%) of the α 1,4-FT activity was possible at 75 μ M of this copolymer. K_i values obtained from Lineweaver–Burke plots were (a) 1.8 μ M for the inhibition of Colo 205 α 1,3-FT activity by 3-sulfoGal β 1,4GlcNAc β -O-A/AA-CP, (b) 4.2 μ M for the inhibition of Colo 205 α 1,3-FT activity by 3-sulfoGal β 1,3GlcNAc β -O-A/AA-CP, and (c) 7.4 μ M for the inhibition of Colo 205 α 1,4-FT activity by 3-sulfoGal β 1,3GlcNAc β -O-A/AA-CP.

When α 1,3-FT activity of HL60 was measured in the presence of increasing concentrations of the copolymers, namely, 3-sulfoGal β 1,3GlcNAc β -O-A/AA-CP or 3-sulfoGal β 1,4GlcNAc β -O-A/AA-CP (see Figure 2), no inhibition of this activity was seen with both copolymers using Gal β 1,4GlcNAc β -O-Al as the acceptor.

When α 1,4-FT activity of the cloned enzyme FT III was measured in the presence of the above copolymers using Gal β 1,3GlcNAc β -O-Al as the acceptor, the copolymer 3-sulfoGal β 1,4GlcNAc β -O-A/AA-CP did not inhibit the α 1,4-FT activity. In fact, there was a small amount of stimulation of this activity with an increase in the copolymer concentration. On the other hand, a gradual decrease in α 1,4-FT activity was noticed with the other copolymer, 3-sulfoGal β 1,3GlcNAc β -O-A/AA-CP. Inhibition reached 56.9% at 12.5 μ M and 84.3% at 62.5 μ M concentrations of this copolymer. K_i for this inhibition was found to be 13.9 μ M.

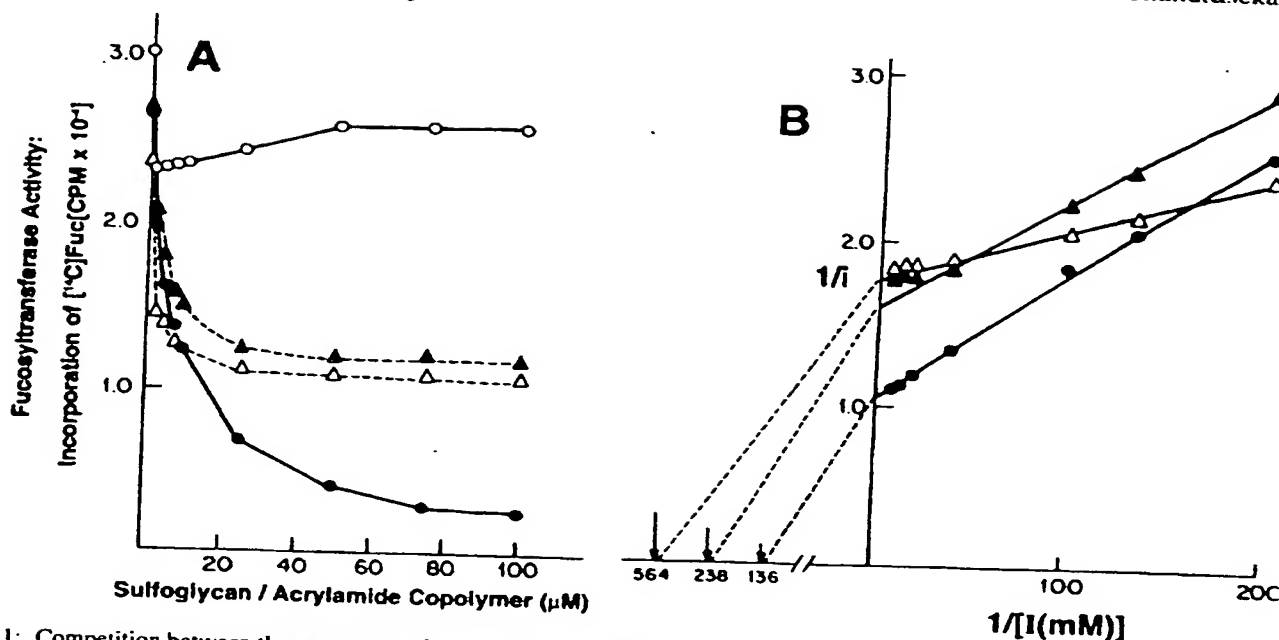


FIGURE 1: Competition between the acceptors and acrylamide-sulfoglycan copolymers for Colo 205 $\alpha 1,3/4$ -FTs. (A) Effect of acrylamide-sulfoglycan copolymers on the $\alpha 1,3$ -L- and $\alpha 1,4$ -L-fucosyltransferase activities of Colo 205: (●) Fuca1,2Gal β 1,3GlcNAc β -O-Al/AA-CP; (○) Gal β 1,3GlcNAc β -O-Al/AA-CP; (Δ) 3-sulfoGal β 1,3GlcNAc β -O-Al/AA-CP. (B) Determination of K_i for the inhibition of Colo 205 $\alpha 1,4$ -FT activity by (●) 3-sulfoGal β 1,3GlcNAc β -O-Al/AA-CP, Colo 205 $\alpha 1,3$ -FT activity by (Δ) 3-sulfoGal β 1,4GlcNAc β -O-Al/AA-CP.

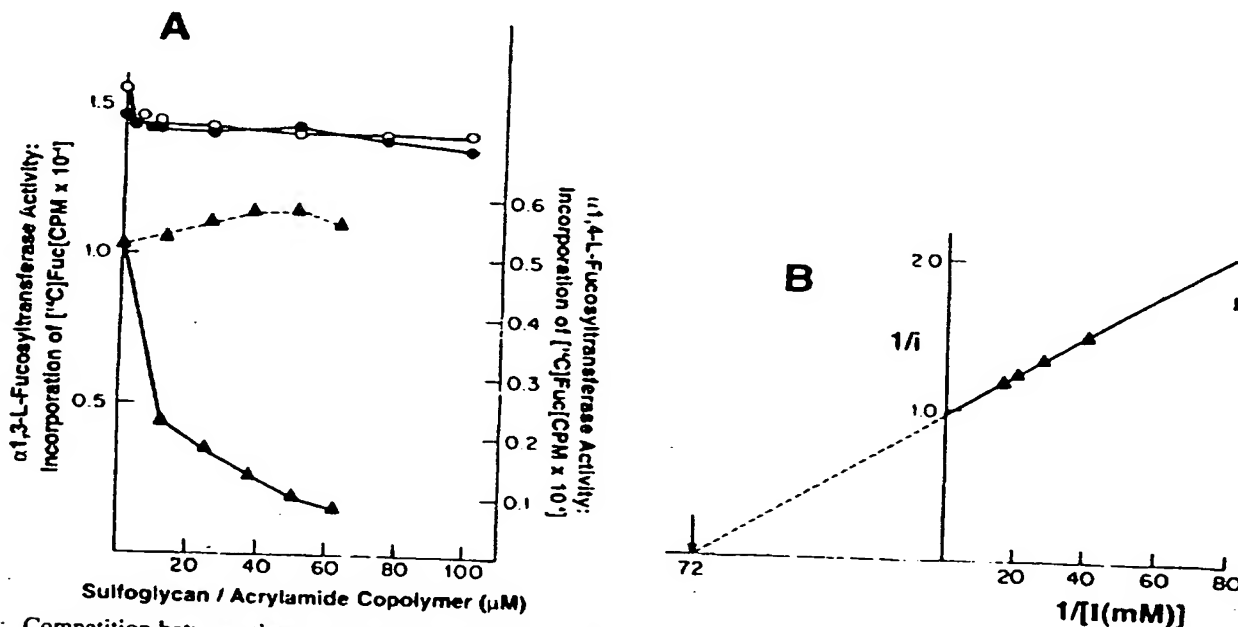


FIGURE 2: Competition between the acceptors and acrylamide-sulfoglycan copolymers for HL 60 $\alpha 1,3$ -FT and the cloned enzyme I ($\alpha 1,4$ -FT). (A) The effect of acrylamide-sulfoglycan copolymers on HL60 $\alpha 1,3$ -FT and FT III $\alpha 1,4$ -FT activity. HL60 $\alpha 1,3$ -FT activity with Gal β 1,4GlcNAc β -O-Al as the acceptor in presence of (●) 3-sulfoGal β 1,4GlcNAc β -O-Al/AA-CP and in presence of (○) 3-sulfoGal β 1,3GlcNAc β -O-Al/AA-CP. FT III $\alpha 1,4$ -FT activity with Gal β 1,3GlcNAc β -O-Al as the acceptor in presence of (Δ) 3-sulfoGal β 1,4GlcNAc β -O-Al/AA-CP. (B) Determination of K_i for the inhibition of FT III $\alpha 1,4$ -FT activity by (Δ) 3-sulfoGal β 1,4GlcNAc β -O-Al/AA-CP.

Recently, Sasaki et al. (1994) reported the expression of both Fuc-T IV and Fuc-T VII in myeloid lineage cells, and they also identified the 3'-sialyl type 2 structure as an exclusive acceptor for Fuc-T VII. The $\alpha 1,3$ -FTs present in CMLN, HL60, B142, and EKVX were shown in the present study to be exclusively active with type 2 acceptors. These enzymes exhibited some activity toward NeuAc $\alpha 2,3$ Gal β 1,4GlcNAc β -O-Bn (7%–13%). It is known from several

studies that 3'-sialyl type 2 does not serve as an acceptor for myeloid type Fuc-T IV. The present study also found that Fuc-T IV was least reactive with this structure compared to its activity with 2'-fucosyl type 2. Thus, results appear to be consistent with the findings of Sasaki et al.

Sasaki et al. (1994) have shown that Fuc-T VII does react with neutral type 2, Gal β 1,4GlcNAc β - structure, BNSF

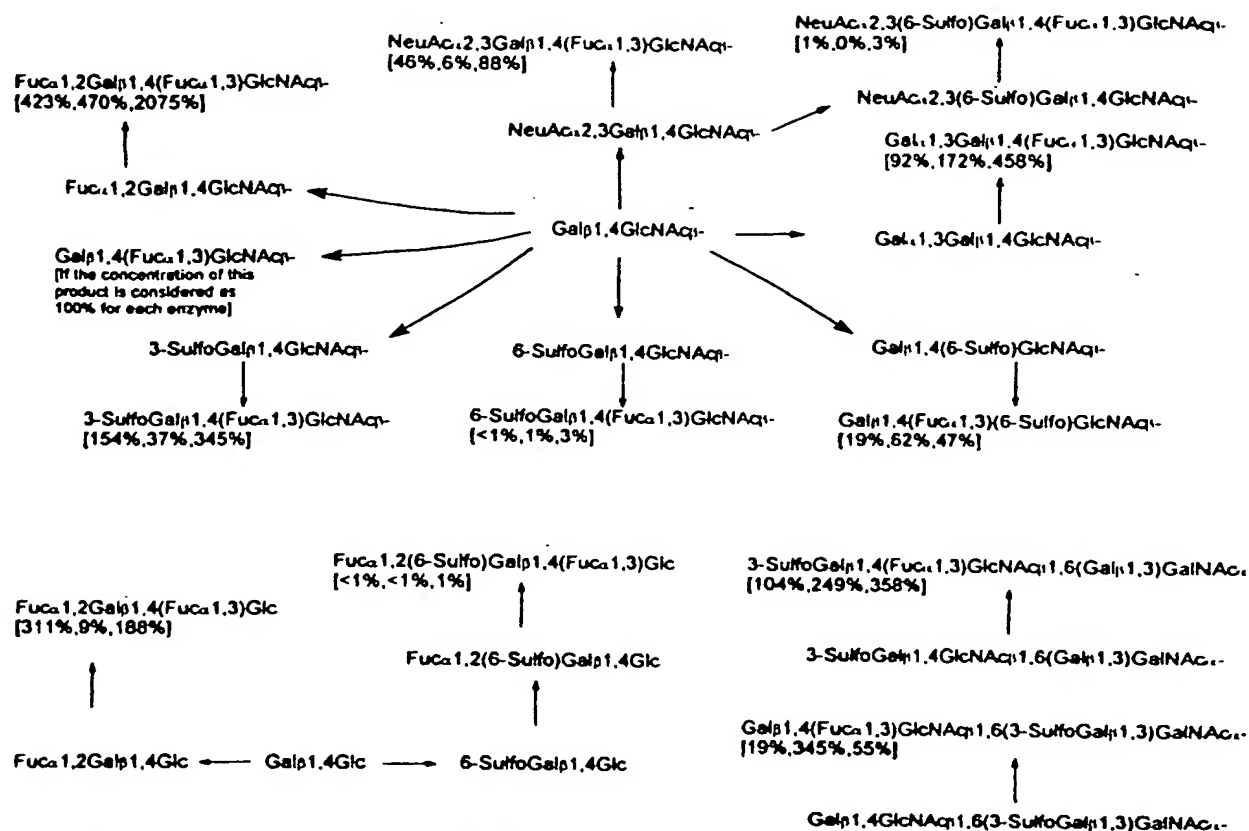


FIGURE 3: Specificities of the cloned enzymes FT III (Lewis type), FT IV (myeloid type), and FT V (plasma type) toward blood group type 2 chain-based structures (The amount of product formed from each compound tested as the acceptor is shown in parentheses as the percent of the activity with the basic structure Galβ1,4GlcNAcβ-1; the three values in the order shown in parentheses are obtained for FT III, FT IV, and FT V, respectively).

activity measured in CMLN, HL60, B142, and EKVX with neutral type 2 acceptors must be attributed to Fuc-T IV. We find that the activities shown by the above FTs with Fuc-α1,2Galβ1,4GlcNAcβ-O-pNP and Galα1,3Galβ1,4GlcNAcβ-O-Bn were almost the same in each case. These results would indicate that Fuc-T is capable of forming Lewis y as well as 3'-α-galactosyl Lewis x at the same rate.

Assuming from the available evidence that Fuc-T IV reacts with neutral type 2 and Fuc-T VII with 3'-sialyl type 2, a structure such as 3'-sulfoLacNAc, which is similar to 3'-sialylLacNAc, is expected to serve as an efficient substrate for Fuc-T VII and not for Fuc-T IV. We also found that with 3'-sulfoLacNAc Fuc-T IV is only 37% active as compared to its 470% activity shown with 2'-fucosylLacNAcβ-O-pNP. On the contrary, the high activity shown by Fuc-T IV toward 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)-GalNAcα-O-Bn would suggest that the enzyme affinity for a particular structure is greatly influenced by the neighboring sugars.

Sasaki et al. found Fuc-T III (Lewis-type), Fuc-T IV (myeloid-type) and Fuc-T VI (plasma-type) to be expressed at a significant level in Colo 205 in ratios of 2.0:0.2:1.1, respectively. They also found levels of Fuc-T IV and Fuc-T VII (3'-sialylLacNAc, an exclusive substrate) in HL60 in a ratio of 1.3:0.8. The absence of Fuc-T III and the presence of Fuc-T VII in HL 60 are also evident from the present study which demonstrated the absence of α1,4-FT activity and the presence of α1,3-FT acting on 3'-sialylLacNAc in HL60. We have shown in an earlier study the occurrence of both Lewis type and plasma type FTs in Colo 205 by

separating the two enzymes through affinity and gel filtration columns (Chandrasekaran et al., 1995). The present study has shown the unique ability of Colo 205 FT to use the copolymer 3'-sulfoLacNAcβ-O-Al/AA-CP as an acceptor. When it was tested as a competitive inhibitor, this copolymer inhibited the α1,3-FT activity of Colo 205 to a great extent but did not inhibit HL60 α1,3-FT. Further, this acceptor was not able to inhibit the α1,4-FT activity of Colo 205 which has inherent α1,3-FT activity. These results indicate the usefulness of this copolymer as an acceptor and as a competitive inhibitor for identifying Fuc-T VI (plasma type) in tissues and cell lines.

The activities of FT III, FT IV, and FT V toward blood group type 2-based compounds as well as the activity of FT III toward blood group type 1-based compounds are depicted in Figures 3 and 4, respectively. For each enzyme the concentration of the product formed from various acceptors is depicted as the percentage of product arising from basic type 1 (Galβ1,3GlcNAcβ-) or type 2 (Galβ1,4GlcNAcβ-). This is also indicated in the figures. These illustrations would enable one to understand the intricate specificities of these enzymes through a direct comparison of their activities with each compound tested as an acceptor and also by a comparison of the activities of each enzyme toward different acceptors. Several unique differences in the specificities of these enzymes were noticeable as listed below:

(A) The participation of FT V is extremely shifted toward the formation of Lewis y (2075%) and 3'-α-galactosyl Lewis x (458%) as compared to the formation of Lewis x (100%).

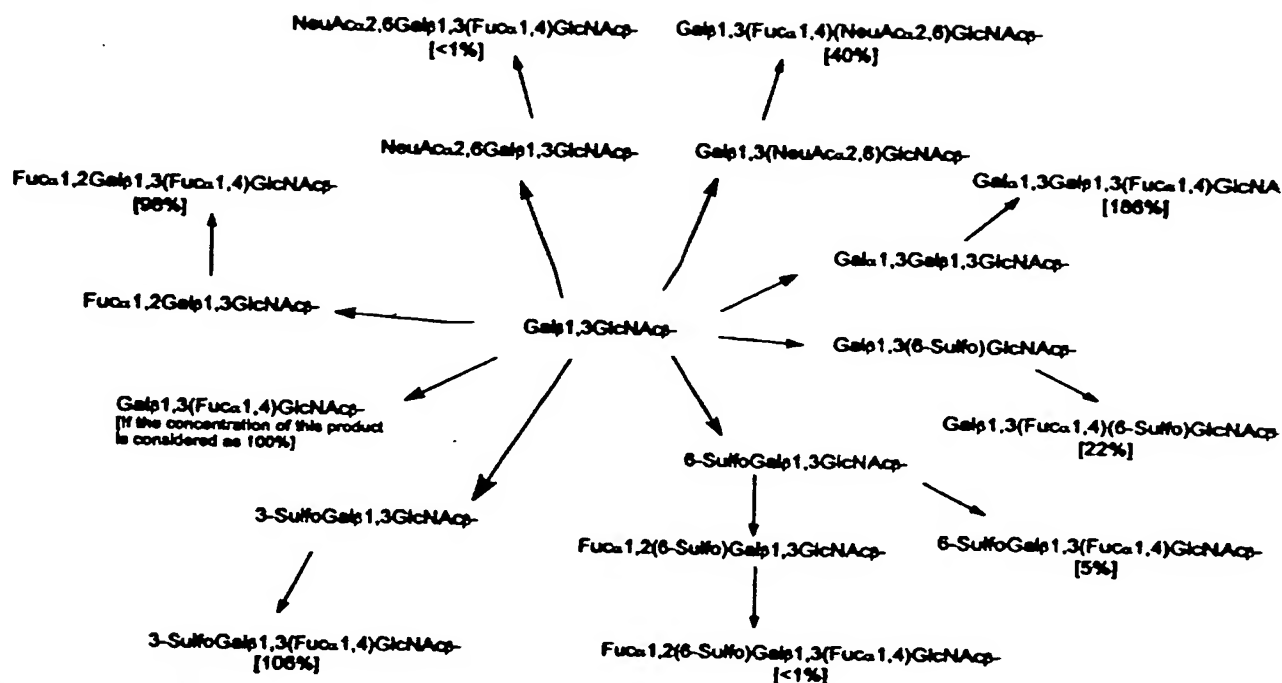


FIGURE 4: Specificities of the cloned enzyme FT III (Lewis type) toward blood group type I chain-based structures. (The acceptor product formed from each compound tested as the acceptor is shown in parentheses as the percent of the activity with the basic acceptor, Gal β 1,3GlcNAc-).

(B) Both FT III and FT V are quite efficient in synthesizing 3'-sialyl Lewis x (46% and 88%, respectively), but they show higher efficiency in forming 3'-sulfo Lewis x (154% and 345%, respectively).

(C) All three enzymes catalyze the formation of 6-sulfo Lewis x, but FT IV appears to be the most efficient (62%).

(D) It is noteworthy that the biosynthesis of either 6'-O-sulfo Lewis x or 3'-sialyl-6'-O-sulfo Lewis x from their immediate precursors does not seem to be catalyzed by these FTs. The latter structure occurs as a part of the carbohydrate moiety in GLYCAM-I (Hemmerich et al., 1995), and on the basis of our observations sulfation appears to be the last step in its synthesis (Chandrasekaran et al., 1995).

(E) Both FT III and FT V are highly efficient in converting 2'-fucosyllactose to 2'-fucosyl-3-fucosyllactose (311% and 188%, respectively), but all three enzymes do not form 2'-fucosyl-6'-sulfo-3-fucosyllactose from the immediate precursor.

(F) FT IV appears to be unique in showing vast preference for forming 3'-sulfo Lewis x (249%) as well as Lewis x (345%), where the precursor structures occur as part of a mucin type chain.

(G) The activities of FT III and FT V with the basic type 1 are ~7 times and 0.5 times those of the basic type 2, respectively, whereas FT IV is almost inactive with type 1.

(H) The acceptor activity of H-type 1 with FT III is not greater than that of the basic type 1, whereas the formation of 3'- α -galactosyl Lewis x is nearly twice.

(I) The most interesting finding is the facile synthesis of Gal β 1,3(Fuc α 1,4)(NeuAc α 2,6)GlcNAc- from Gal β 1,3-(NeuAc α 2,6)GlcNAc- by FT III (40%).

(J) Either C-6 sulfation or C-6 sialylation of Gal in type I leads to almost complete loss of acceptor activity toward FT III.

(K) On C-3 sulfation of Gal or C-6 sulfation of C in type I, the acceptor activities became 106% and 188%, respectively, toward FT III.

Thus, the above findings on the specificities of these enzymes would serve as a sound basis to explain the role of these enzymes in the expression of biologically relevant carbohydrate ligands and antigenic determinants.

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